1998

Purification and Antioxidant Properties of Rice Bran (Gamma)-Oryzanol Components.

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PURIFICATION AND ANTIOXIDANT PROPERTIES OF RICE BRAN γ-ORYZANOL COMPONENTS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

In

The Department of Food Science

by
Zhimin Xu
B.S., East China University of Science and Technology, 1985
M.S., Louisiana State University, 1994
May 1998

ACKNOWLEDGEMENTS

The author wishes to express his most sincere appreciation to his major professor, Dr. J. Samuel Godber, Professor, Department of Food Science, for his precious and patient guidance, encouragement, and advice during the investigation and the preparation of this dissertation.

Appreciation is also extended to Dr. Maren Hegsted, Professor of the Department of Human Ecology, Dr. Simon M. Shane, Professor of the Department of Epidemiology, Dr. Micheal J. Keenan, Associate Professor of the Department of Human Ecology, and Dr. Leslie C. Plhak, Assistant Professor of the Department of Food Science for their advice, instructions, and for their serving as members of the examination committee. Thanks are also extended to Dr. Douglas L. Park, Head of the Department of Food Science, for his support during this research.

The author would like to express his deepest gratitude to his parents and sister for their tremendous encouragement, support, and love during this endeavor.

Most of all, the author wishes to thank his wife, Mrs. Zhiyi Liang, for her endless and priceless inspiration, understanding, sacrifice, and love. He would also like to thank his son, Leon Xu, for his wonderful love. It is to them that this work is dedicated.

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ABSTRACT

A high purity γ-oryzanol was obtained from crude rice bran oil using a normal phase preparative scale HPLC. It was used in implementing a reverse phase HPLC method for separating each individual component of γ-oryzanol present in rice bran oil. Ten fractions were isolated in the reverse phase HPLC method. The ten components of γ-oryzanol were identified. Three of these, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, were major components of γ-oryzanol.

The yield of γ-oryzanol without saponification was significantly higher than that with saponification in solvent extraction. The solvent mixture with 50% hexane and 50% isopropanol (v/v.) at an temperature of 60°C for 60 min produced the highest yield of γ-oryzanol among test solvents and conditions. However, the yield of γ-oryzanol in supercritical fluid extraction under temperature 50°C, pressure 680 atm, and time 25 min was approximately four times higher than the highest yield of solvent extraction. Also, high concentration of γ-oryzanol in extract (50 - 80%) was obtained by collecting the extract between 15 - 20 min of extraction under this optimized condition.

A quantitative method for determining four hydroperoxide isomers of linoleic acid, 9HPODE(t,c), 9HPODE(t,t), 13HPODE(c,t), and 13HPODE(t,t), using a normal phase HPLC with a UV detector was developed. The sensitivity and specificity of the HPLC method were sufficient to measure the production of the four hydroperoxide isomers when linoleic acid was incubated at 37°C with constant air flow. α-Tocopherol and ferulic acid showed significant antioxidant activity via low productions of

hydroperoxides, compared to control. Based on the relative production of each hydroperoxide, the mechanisms of antioxidants, scavenging singlet oxygen or scavenging peroxyl free radicals, were also determined. Different profiles of production of each hydroperoxide in the linoleic acid model with α-tocopherol and ferulic acid indicated that they possess different mechanisms of antioxidation.

The three components of γ-oryzanol evidenced significant antioxidant activity when they were mixed with linoleic acid in a molar ratio of 1:100 and 1:250 but not in a molar ratio of 1:500. The antioxidant mechanisms of the three major components of γ-oryzanol were similar to that of ferulic acid.

CHAPTER 1

INTRODUCTION

γ-Oryzanol was first extracted from rice bran oil and considered to be a single component. Later it was determined to be a mixture of ferulate (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols. Major components of γ-oryzanol were identified as ferulate esters of cycloartenol, 24-methylene cycloartanol, and campesterol (Norton, 1995; Rogers et al., 1993; Seitz, 1989).

γ-Oryzanol was considered to be an important fraction, along with tocotrienols and unsaponifiables in the hypocholesterolemic property of rice bran oil (Seetharamaiah and Chandrasekhara, 1988, 1989; Sharma and Rukmini, 1986). γ-Oryzanol was also reported to possess antioxidant activity in stabilizing lipid (Duve and White, 1991; Sonntag, 1979). This antioxidative property may help to protect against various vascular diseases and cancers in biological systems that generally result from cell damage caused by free radicals. However, the efficiency and mechanism of the antioxidant capacity of γ-oryzanol fractions are less understood. Therefore, the purpose of this study was to investigate the antioxidant properties of the major γ-oryzanol components. γ-Oryzanol components antioxidant activities will be compared with that of α-tocopherol and ferulic acid under identical experimental conditions. Results of this study may help us to understand the antioxidant properties of γ-oryzanol components and clarify the potential nutritional value of rice bran.

Extraction, purification, separation, identification, and quantification of the γ-oryzanol components would be necessary before a reliable nutrition study could be

performed. Traditional solvent extraction with saponification was used in extraction of γ-oryzanol from rice bran. However, the effects of solvent polarity and saponification on efficiency of γ-oryzanol extraction from rice bran have not been reported. Currently, supercritical fluid extraction is being suggested for lipid extraction. It may have higher efficiency and greater advantage than the traditional solvent extraction in γ-oryzanol extraction. This study investigated the effects of solvent polarity and saponification, and optimum conditions of supercritical fluid extraction in γ-oryzanol extraction.

Separation and identification of components of γ-oryzanol from rice bran using analytical reverse or normal phase HPLC methods have been reported (Diack and Saska, 1994; Evershed et al., 1988; Norton, 1995; Rogers et al., 1993; Seitz, 1989). Although the three major components, ferulate esters of cycloartenol, 24-methylene cycloartanol, and campesterol, were identified in most of these reports using a reverse phase HPLC method, other components were still unknown or identified tentatively due to low levels of these minor components in rice bran and incomplete separation. Furthermore, since standards of these components are not available, the quantitative methods for the individual components of γ-oryzanol in those reports were not adequate. In this study, improved separation, identification, and quantification of individual component of γ-oryzanol were obtained and purification of the major components of γ-oryzanol using preparative scale liquid chromatography was developed.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Nutritional values of rice bran

Rice is one of the major crops in Louisiana. It was estimated that in Louisiana about 620,000 acres of rice were planted and 1.44 million tons rough rice was produced in 1992, which accounted for 16% of the total U.S. rice production. Rice products are important agronomic commodities for Louisiana agricultural business. The value-added processing of rice by-products, such as rice bran, could potentiate more efficient utilization of rice resources and improve economic return from the Louisiana rice crop.

Rice as harvested is referred to as paddy where the kernel is fully sealed by the rice hull. The hull is removed to yield brown rice, in the first milling operation, which is still enclosed by a bran layer. The outer layer is removed from the rice kernel to yield white rice in the second milling operation. The separated brown layer in this stage is called rice bran. The amount of rice bran is 8-12% of the total weight of brown rice. It is an underutilized by-product. Most of it is utilized only as livestock feed or must be disposed of.

The major components of rice bran are approximately 12 - 22 % oil, 11 - 17 % protein, 6 -14 % fiber, 10-15 % moisture, and 8-17 % ash (Saunders, 1990). Rice bran also is rich in a variety of vitamins and minerals. These include thiamin, niacin, vitamin E, phosphorus, potassium, magnesium, and silicon. Rice bran oil and dietary fiber have

been shown to most greatly contribute to the potential of rice bran in lowering serum cholesterol level.

The lipid in rice bran contains oleic acid (38.4 %), linoleic acid (34.4 %), and linolenic acid (2.2 %) as unsaturated fatty acids, and palmitic (21.5 %) and stearic acid (2.9 %) as saturated fatty acids. The unsaponifable fraction of rice bran (4.2 %) includes tocopherols and tocotrienols (0.08 %), γ-oryzanol (1.6 %), and squalene (0.32 %) (Hemavathy and Prabhakar, 1987; Rukmini and Raghuram, 1991). γ-Oryzanol was considered to be an important fraction, along with tocotrienols and other unsaponifiables in the hypocholesterolemic property of rice bran oil (Rukmini and Raghuram, 1991; Saunders, 1990; Seetharamaiah and Chandrasekhara, 1988, 1989; Sharma and Rukmini, 1986). The fiber in rice bran contains both insoluble and soluble fiber. Which have been reported to significantly lower serum cholesterol (Slavin and Lampe, 1992).

High levels of tocopherols and tocotrienols in rice bran are important in protection against oxidation of rice bran oil. γ-Oryzanol was also reported to possess antioxidant activity in stabilizing lipid (Duve and White, 1991; Ramarathnam et al., 1989; Sonntag, 1979). However, the efficiency and mechanism of antioxidation of the γ-oryzanol fraction were not discussed in these reports.

2.2. γ-Oryzanol of rice bran

γ-Oryzanol was first extracted from rice bran oil and considered to be a single component. Later it was determined to be a mixture of ferulate (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols. Major components of γ-oryzanol

were identified as cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate (Figure 1). They occupy about 70% of the total weight of γ-oryzanol in rice bran (Norton, 1995).

2.3. Extraction, purification, separation, identification, and quantification of components of γ-oryzanol

2.3.1. Solvent extraction

Since γ-oryzanol is readily dissolved in organic solvents, hexane was typically used in extraction of γ-oryzanol from rice bran (Diack and Saska, 1994; Rogers et al. 1993; Seitz, 1989; Norton, 1994). However, all components of γ-oryzanol contain an alcohol group in the ferulate portion, which brings about a relatively low polarity of these components. They may also be soluble in low polar solvents, such as isopropanol and ethyl acetate, in addition to the non-polar solvents, hexane or heptane. The solvent strength of extraction may affect the extractability of γ-oryzanol from rice bran. The effects of various solvents on the yield of γ-oryzanol in extraction have not been reported.

Another unclear factor in extraction is the effect of saponification on the efficiency of extraction. In previous studies, saponfication was performed prior to the solvent extraction (Diack and Saska, 1994; Rogers et al. 1993; Seitz, 1989; Norton, 1994). Saponification, which is important for reducing interfering lipids and for breaking down the matrix of rice bran for improved recovery of extraction, may have a negative effect in the extraction of γ-oryzanol. It is possible that the ester bond between the ferulate and triterpene components of γ-oryzanol also is broken down under alkali

condition. This could result in decomposition of γ-oryzanol and decrease the yield of extraction. The effect of saponification on yield of γ-oryzanol in solvent extraction has not been reported.

2.3.2. Supercritical fluid extraction

Supercritical fluid extraction (SFE) of lipid has received attention as an alternative to organic solvent extraction. Supercritical carbon dioxide has been shown to be an ideal solvent for extracting certain lipids. Supercritical carbon dioxide extraction in place of solvent extraction was studied intensively (Bhaskar et al., 1993; Garcia et al., 1996; Froning et al., 1990; List et al., 1993; Merkle and Larick, 1993; Tsuda et al., 1995). Carbon dioxide is changed to supercritical fluid beyond the supercritical point (73 atm, 31°C). Supercritical carbon dioxide has unique solvating characteristics, since supercritical fluids have lower viscosity and diffuse more rapidly into a sample matrix than conventional solvents. Supercritical carbon dioxide is non-toxic, non-flammable, and low cost in chemical extraction, compared to solvents used in traditional extraction. These advantages may make it preferred in food and pharmaceutical industries.

Most studies of SFE on lipid extraction focused on yield of extractable material. The supercritical fluid pressure, temperature, and time were optimized for obtaining as much extract from sample as possible. Garcia et al. (1996) reported that the condition of the highest yield of extract from rice bran in their study was the highest pressure and temperature allowable in their system (28MPa and 70°C) and the yield was only 16-60% of that obtained by solvent extraction with hexane. Tsuda et al. (1995) indicated

that the yield of extract from seed coat decreased when temperature increased above 40°C at constant pressure.

Since the principle of SFE is similar to solvent extraction, extractability of a compound depends on the relationship of its chemical characteristic and strength of supercritical fluid. For each compound there exists a unique extractability under different condition of supercritical fluid that is a combination of factors, such as extraction temperature, supercritical fluid pressure, and extraction time. In theory, components in a sample are extracted in an ordered manner from sample matrix under ideal conditions of supercritical fluid extraction. As supercritical fluid removes components during extraction, fractionation of the extract becomes possible and convenient. Therefore, supercritical fluid extraction can be used not only in extracting components from sample but also in fractionating the components at the same time. Fractionating components of interest during extraction has the advantage of gaining a higher concentration of the component than is possible with traditional solvent extraction. This can reduce the cost and time of purifying specific components of interest from the extract inherent in traditional solvent extraction. Application of supercritical fluid for the purpose of extraction of γ-oryzanol has not been reported in previous studies.

2.3.3. Low pressure chromatography in purification of γ-oryzanol

After solvent extraction, crude rice bran oil containing a large amount of various lipids, besides γ-oryzanol, is obtained. To purify γ-oryzanol from interfering lipids in crude oil, a low pressure chromatography technique is usually applied. Boven et al.

(1997) reported that an aluminum oxide and silica gel column were used to isolate the free sterol and minor compounds from rice bran oil, respectively. Kuroda et al. (1977) used a silica gel column to obtain the sterol lipid fraction from rice bran oil. Hexane mixed with another relatively high polarity solvent was used to flush the low pressure column. γ-Oryzanol was fractionated and collected when it eluted from the column. Most interfering lipids were removed. Because of low resolution in low pressure column chromatography, γ-oryzanol was only partially purified and individual components of γ-oryzanol could not be isolated.

2.3.4. Preparative scale HPLC in purification and isolation of individual component of γ-oryzanol

γ-Oryzanol may be purified on a relatively large scale using preparative scale HPLC. Application of preparative scale HPLC in purifying and isolating individual components of γ-oryzanol has not been studied. Preparative scale HPLC has been gaining attention over the past few years, particularly in the pharmaceutical industry. This type of chromatography makes use of stainless steel columns able to withstand high pressure (up to 1500 psi.) and packed with small-size particles (10 to 30 μm). The preparative scale HPLC is similar to analytical HPLC, except for the size of the column and capability of the mobile phase pump. It can be loaded with a relative large sample with high column efficiencies. However, the preparative scale HPLC is considered to be a very expensive technique, difficult to use, not really applicable on a large scale, and limited to very high added value products (Ganetsos and Barker, 1993). The amount of sample loading should be as great as possible within required resolution. Otherwise, preparative HPLC results in high cost per product unit.

2.3.5. Separation of components of γ-oryzanol

Initially, γ-oryzanol was analyzed from rice bran oil using thin layer chromatography (TLC) technology. It was isolated as a single compound due to the limitation of resolution of TLC, even though γ-oryzanol is a mixture of ferulate esters of triterpene alcohols.

Lately, high performance liquid chromatography (HPLC) has been applied in the study of γ-oryzanol. Normal and reverse phase HPLC analyses were performed to separate the components of γ-oryzanol. In normal phase HPLC, γ-oryzanol was separated into two fractions that were mixtures of two or more individual components of γ-oryzanol (Diack and Saka, 1994). Compared to normal phase HPLC, reverse phase HPLC had higher resolution in separation. Usually, four fractions can be obtained (Norton, 1995; Rogers et al., 1993). However, some components of γ-oryzanol still were not separated individually. This limits identification and quantification of each of the components of γ-oryzanol. Therefore, the method of separating minor components of γ-oryzanol needs to be improved.

2.3.6. Identification and quantification of γ-oryzanol

Gas chromatography-mass spectrometry (GC-MS) is a convenient method to identify structure of unknown compounds. From mass spectrum, the information of molecular weight and main structural aspects of compounds can be accessed. Components of γ-oryzanol are difficult to mobilize with the gas phase in a GC column since their temperatures of volatilization are above 400°C. They have to be split into relatively small molecule (triterpene alcohol and ferulic acid) or estertified to reduce the

attractive force between molecules by modifying their polar groups and lowering their temperatures of volatilization. These components possess ester bonds that connect the triterpene alcohol and ferulic acid. They can be saponified in alkali solution to break down their ester bond. Triterpene alcohol and ferulic acid are formed during saponification. Since the only difference between γ-oryzanol components is in the structures of their triterpene alcohols, they can be identified according to their triterpene alcohol structures in the mass spectrums. Triterpene alcohols are not easily volatilized compounds either. They are usually derivatized to less polar compounds before analysis in the GC-MS. Norton (1995) and Rogers et al. (1993) reported that bis(trimethylsily)-trifluoroacetamide (BSTFA) was used to derivatize the triterpene alcohol to form a volatile trimethylsilyl (TMS) ether derivative after the triterpene was produced in the saponification.

Using this method, three of four fractions were obtained in reverse phase HPLC and identified as cycloartenyl ferulate, 24-methylene cycloartanly ferulate and campesteryl ferulate (Norton, 1995; Rogers et al., 1993). These are the major components of γ-oryzanol. However, some fractions remained unidentified. They may be mixtures of more than one component of γ-orzyanol due to unsatisfactory separation, which makes identification of these individual components difficult.

In Norton (1995) and Rogers et al. (1993), one of the major components of γ-oryzanol was used as a standard for all fractions in quantification since high purity standards for each component were not available commercially and were difficult to prepare. Thus, it was not deemed to be a satisfactory quantification method.

2.4. Oxidation and antioxidation

2.4.1. Principle of lipid oxidation

The process of autoxidation consists of three steps, initiation, propagation, and termination. In the initiation step, radicals are produced. These radicals react with unsaturated fatty acids to form lipid hydroperoxides and generate other free radicals in the propagation step. Reactions during propagation constitute a chain reaction until the termination step. In the termination step, one radical reacts with another radical that pairs odd electrons and forms a non radical product. These three steps are listed below.

Initiation: $LH \rightarrow L\bullet + H\bullet$

$LOOH \rightarrow LO\bullet + HO\bullet$

$2LOOH \rightarrow LO\bullet + LOO\bullet + H_2O$

Propagation: $L\bullet + O_2 \rightarrow LOO\bullet$

$LOO\bullet + LH \rightarrow LOOH + L\bullet$

Termination: $LOO\bullet + LOO\bullet \rightarrow$ non radical product

$LOO\bullet + L\bullet \rightarrow$ non radical product

$L\bullet + L\bullet \rightarrow$ non radical product

Lipid oxidation produces hydroperoxides, the primary oxidation products, which are colorless and odorless. These products are labile and readily produce a number of secondary products, such as alkanes, alcohols, aldehydes, and acids. Some of the secondary products are more odor-active and volatile. Therefore, secondary products

contribute undesirable sensory characteristics to food. In the body, the primary products also affect functions of cell membranes and many of the secondary products are highly reactive and react with the body's biological components, such as protein, DNA, and lipids. This results in a number of diseases and tissue injuries, such as those of the lungs, heart and cardiovascular system, kidneys, liver, gastrointestinal tract, blood, eye, skin, muscle, brain, and with the processes of aging, mutagenesis, and carcinogenesis (Foote, 1968; Gollnick, 1968; Nawar, 1985).

2.4.2. Effect of oxygen and temperature on lipid oxidation

Oxygen is an important factor inducing prooxidant states of lipids. The most activated species of oxygen is singlet oxygen ($^1O_2$). The electronic structure of the activated form of oxygen facilitates its activity with lipids (Halliwell and Gutteridge, 1989). This step is listed below.

$$^1O_2 + LH \rightarrow LOOH$$

Temperature is another important factor affecting oxidation of lipids. The rate of oxidation increases with temperature. Oxidation occurs via the initial formation of hydroperoxides. High temperatures can cause much isomerization to take place and produce a variety of secondary or breakdown products (Parkin and Damodaran, 1993).

2.4.3. Principle of antioxidation

Generally, an antioxidant is a compound that can terminate the free radical chain reaction by donating hydrogen or electrons to free radicals and convert them to more

stable products. The general mechanisms for antioxidants have been reported extensively.

First, antioxidants react with free radicals of lipid to inhibit the propagation step. This is called scavenging free radicals.

$$AH + L\bullet \rightarrow A\bullet + LH$$

$$AH + LOO\bullet \rightarrow A\bullet + LOOH$$

$$AH + LO\bullet \rightarrow A\bullet + LOH$$

Some antioxidants react with singlet oxygen to prevent its reaction with lipid. This step is called scavenging singlet oxygen.

$$AH + {}^1O_2 \rightarrow A\bullet + O_2$$

The free radical antioxidants may further react with a free radical of lipid to produce original antioxidants, degraded antioxidants, and peroxy antioxidant compounds.

$$AH\bullet + AH\bullet \rightarrow AH + AH'$$

$$AH\bullet + LO\bullet \rightarrow LOAH$$

$$A\bullet + LOO\bullet \rightarrow LOOA$$

2.5. Natural antioxidants

Antioxidants from natural sources include phenolic and polyphenolic compounds, chelators, antioxidant vitamins and enzymes. They are involved in the control of food autooxidation and rancidity. Also, they are associated with reducing risk of cardiovascular diseases, cancer, cataracts, and other diseases of aging.

Recently, the use of natural antioxidants has been emphasized because of increasing limitations on the use of synthetic antioxidants, which have been suggested to have health implications. In general, natural antioxidants are preferred by consumers because they are considered safe. Rajalakshmi and Narasimhan (1995) listed some of the advantages and disadvantages of natural antioxidants (Table 1).

Table 1. Advantages and disadvantages of natural antioxidants

Advantages	Disadvantages
1. Readily accepted by the consumer, as considered to be safe and not a "chemical"	1. Usually more expensive if purified and less efficient if not purified.
2. No safety tests required by legislation if a component of a food that is "generally recognized as safe" (GRAS)	2. Properties of different preparations vary if not purified
	3. Safety often not known.
	4. May impart color, aftertaste, or off-flavor to the product.

Many reports have been published on the identification of naturally occurring antioxidants from plants, animals, microbial sources, and other sources. Studies have led to the identification of active ingredients and antioxidant components of natural compounds, such as rosemary, green tea, sesame seed, canola seed, and ginger (Burton

and Ingold, 1984; Teral, 1989; Chimi et al., 1991; Marinova and Yanishieva, 1994). Most natural antioxidants are flavonoids and related compounds in plant extracts and phenolics in spices and herbs (Pratt, 1991).

Flavonoids with antioxidative function exist in fruits and vegetables, oilseeds, tomatoes, onions, etc. and phenolic acids with antioxidant activity occur widely in oilseeds and leaf extracts. The various active components identified in mustard seeds and rapeseeds include cinnamic, ferulic, caffeic, sinapic, salicylic, and vanillic acids (Kozlowska et al., 1990). Phenolic acids with antioxidant properties have also been reported in soybeans, cottonseed, and peanuts.

Ferulic acid is a part of the structural components of γ-oryzanol. Ohta et al. (1994) identified ferulic acid sugar esters as active components in corn bran hemicellulose fragments. Activities of ferulic acid sugar esters were stronger than that of free ferulic acid in the microsomal lipid peroxidation system.

Another part of the structural components of γ-oryzanol, sterol (triterpene), also was found to have antioxidative activity. Takagi and Iida (1980) reported that the antioxidative activity of canary seed extract (*Phalaris canariensis*) was due to various sterols such as gramisterol, cycloartenol, sitosterol, campesterol, and triterpene alcohol ester of caffeic acid.

Antioxidative function of γ-oryzanol have been reported (Duve and White, 1991; Sonntag, 1979). The phenolic hydroxyl group in the ferulate esters of γ-oryzanol may be responsible for its antioxidative function. Additional research is needed to understand the nature of antioxidant function in γ-oryzanol.

2.6. Methods of evaluation of antioxidant activity using lipid systems

Oxidative stability of lipids with addition of an antioxidant can be used to evaluate antioxidant activity. Oxidative products are produced while lipids are oxidized under a constant artificial condition. The number of oxidative products indicates the extent of oxidation. When an antioxidant is in the lipid system, the greater amounts of oxidative products indicate lower antioxidant activity. The oxidative products are usually quantified by chemical, instrumental, and sensory methods. Generally, there are three methods for preparing artificial environmental conditions of lipid oxidation, accelerated stability, chemical generation, and enzymatic generation methods.

2.6.1. Accelerated stability methods

Accelerated stability methods are extensively used in evaluating antioxidant activity of a compound. These tests are performed by increasing temperature and oxygen concentration in a lipid system. Frankel (1991) reviewed and reported the limitations of these tests. Reliable results were obtained when the test was done at room temperature. But the disadvantage of this test was that it was time consuming and had low sensitivity in monitoring oxidative products due to lower concentration. When high temperature was used in the test, more numerous disadvantages occurred and affected the results even though the test time was short. These disadvantages are: 1) the solubility of oxygen decreases at elevated temperatures; 2) side reactions such as polymerization and cyclization become important and may not be relevant to normal room temperature; 3) most antioxidants are subject to significant decomposition at high temperature.

2.6.2. Chemical generation methods

Chemical systems to generate oxygen radicals are reported extensively. Sources for OH free radicals comprise Fenton systems in aqueous solutions (Puppo, 1992) or specific generation systems in organic solvents (Grant et al., 1984; Tezuka, 1988). However, the Fenton system is not clearly understood as reactions of OH free radicals or the strongly oxidizing ferryl species can not be discriminated on the basis of kinetic or analytical data (Rush and Koppenol, 1986; Rush et al., 1986). Niki (1990) stated that peroxyl radicals result from oxygen attachment to alky radicals formed after thermolysis of a number of azo compounds. This system has been applied to study flavonoid antioxidants (Ariga and Hamano, 1990). Chemical generation of alkoxyl radicals via the organic Fenton reaction is a poor system for kinetic studies due to the multitude of radicals formed (Greenley and Davies, 1992).

2.6.3. Enzymatic generation methods

Enzymatic sources include xanthine oxidase (Cotelle et al., 1992) or the NADPH oxidase of phagocytes (Pagonis et al., 1986), which generate predominantly singlet oxygen. These enzymatic reactions are accompanied by production of unknown levels of H_2O_2. The H_2O_2 may form unpredictable levels of OH free radicals by reacting with trace metal ions in the Fenton reaction. Lipoxygenase usually generates fatty acid peroxyl radical (Aziz et al. 1971; Grosch and Laskawy, 1979). Since these are produced in close proximity to the prosthetic site, they may not be freely diffusible and would not be capable of reacting with antioxidants.

2.7. Measurement of oxidative products

2.7.1. Chemical methods

Various chemical methods have been developed to measure hydroperoxides, hydroxides, free fatty acids, and decomposition products, especially aldehydes, short chain free fatty acid, and ketones. Peroxide value (PV) is the most common measure of oxidative rancidity. The sample is reacted with a saturated aqueous solution of potassium iodide, and the iodine liberated by the peroxides is titrated with a standard solution of sodium thiosulfate (AOCS, 1994).

The thiobarbituric acid (TBA) test also is widely used in monitoring oxidation of lipids. It is based on the color reaction of TBA with malonaldehyde. The colored solution is measured at a wavelength of 532 nm. The Kreis test is another colorimetric method based on the reaction of phloroglucinol with epoxyaldehydes and malonaldehyde. The anisidine value depends on the reaction of aldehydes, especially 2-alkenals, with *p*-anisidine, and the reaction products are measured at 350 nm. The carbonyl value is a measure of the carbonyl compounds formed during oxidation. Volatile carbonyl products are converted to 2,4-dinitrophenylhydrazone derivatives in the assay (Henick et al., 1954)

The advantages of these methods are that they are fast and simple. However, the disadvantages are low accuracy and specificity, and poor repeatability.

2.7.2. Direct spectrophotometric methods

Since hydroperoxides and conjugated dienes have stronger absorption in the UV region at 234 nm, direct spectrophotometric methods can be used. The extent of

formation of hydroperoxides is indicated by the increased absorbency measured at a wavelength of 234 nm. However, besides hydroperoxides, many compounds are produced during oxidation or originally existed in the lipid system that also have stronger absorption at this wavelength. This causes the method to lack sensitivity and specificity.

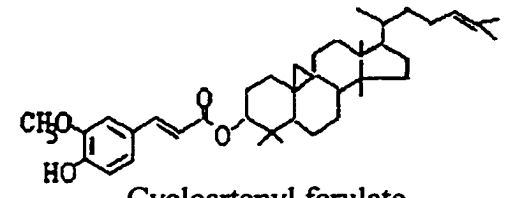
Recently, electron spin resonance (ESR) spectroscopy has been used extensively in detecting free radicals. It is based on the absorption of microwave energy emitted by the promotion of an electron to a higher energy level when the sample is placed in a variable magnetic field. The ESR technique is more suitable in biological and food systems. Compared to traditional spectrophotometric methods, sample matrix usually does not largely affect results in the ESR method. The ESR method has high sensitivity and employs simple sample treatment, but is expensive and not commonly available in labs.

2.7.3. Chromatographic methods

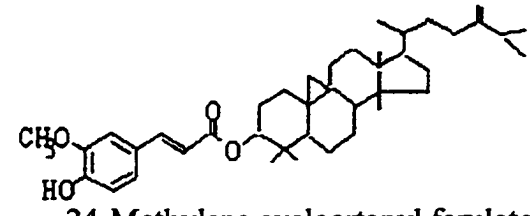
Gas chromatographic (GC) methods are widely used for the measurement of volatile compounds, either by headspace analysis or by direct injection. Oxidation products of lipids, such as 2-heptanal, 2-decanal, 2,4-decadienal, 2,4-heptadienal, hexanal and pentane, are measured by GC. The disadvantage of GC is that the oxidation of lipids is accelerated in sample preparation and chromatography under high temperature. This affects the reliability of results.

High performance liquid chromatography is another chromatography technique to measure hydroperoxides, hydroxides, and secondary oxidation products. The

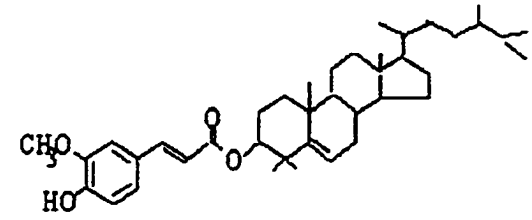
advantage of the HPLC method is that decomposition of the relatively labile hydroperoxides and other oxidative products is reduced. The difficulty in using HPLC methods is poor sensitivity in detection due to low concentration of hydroperoxides and other products. UV and post-column reaction detection systems are usually used in these methods. For the UV detector, the wavelength is set at 234 nm, which is the maximum absorbency for polyunsaturated fatty acid hydroperoxides (DeMeyer et al., 1991). In DeMeyer et al. (1991), two hydroperoxides, 13-hydroxyoctadecadienoic and 15-hydroxyeicosatrienoic acid, were quantified using a reversed phase column. However, quantification of four hydroperoxides of linoleic acid using HPLC have not been reported. For post-column reaction detection systems, certain chemicals are used to react with these oxidation products that elute from the HPLC column to produce high absorbency substances (Akasaka, 1993; Miyazawa et al., 1994; Yamamoto, 1994). These methods have higher sensitivity than direct UV detection.



Cycloartenyl ferulate



24-Methylene cycloartanyl ferulate



Campesteryl ferulate

Figure 1. Molecular structure of cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate.

CHAPTER 3

MATERIALS AND METHODS

3.1. Separation and identification of components of γ-oryzanol

3.1.1. Chemicals and materials

All solvents were HPLC grade. Hexane was from Curtin Matheson Scientific Inc. (Houston, TX). Ethyl acetate was from EM Science (Gibbstown, NJ). Methanol and dichloromethane were from Mallinckrodt Baker Inc. (Paris, KY). Acetonitrile and acetic acid were from Fisher Scientific Inc. (Fair Lawn, NJ). Ascorbic acid and sodium sulfate anhydrous were from Sigma (St. Louis, MO). Rice bran was a gift from the Riviana Rice Mill (Abbeville, LA).

3.1.2. Extraction of crude oil

Twenty five gram of rice bran were placed in a 500-mL round bottom flask with 1 g ascorbic acid, 35 mL hexane, and 15 mL ethyl acetate. The flask was attached to a rotary evaporator (RE121, Brinkmann Inc. Switzerland) and placed in a 60°C water bath for 40 min with 180 rpm. Then 25 mL distilled water was added to the flask. The flask was placed on the rotary evaporator with the same temperature water bath and rotation speed for 10 min. Solvent and rice bran residual were separated by filtration. Rice bran residual was extracted a total of three times using this process. The extracts of the three extractions were pooled and centrifuged at 4000×g for 10 min. The organic solvent

layer was transferred to a 200-mL Erlenmeyer flask. Crude oil was obtained after the solvent in the flask was evaporated in the rotary evaporator under vacuum at 60°C.

3.1.3. Semi-purification of γ-orzyanol in a low pressure silica column

A glass column (2.5 cm × 25 cm) packed with 20 g silica (Grade 62) (EM Industry Inc. NJ) was used to remove the triglycerides and other lipids. The solvent consisted of hexane and ethyl acetate. The first step of the clean-up employed 50 mL of the solvent (hexane : ethyl acetate = 9:1) for flushing through the column. In the next step, 50 mL of the solvent (hexane : ethyl acetate = 7:3) was flowed through the column and the eluant was collected. Then the column was washed with 50 mL of hexane : ethyl acetate (5:5) and the semi-purified γ-oryzanol was obtained after the solvent was evaporated.

3.1.4. Purification of γ-oryzanol using a preparative scale normal phase HPLC

The preparative HPLC system consisted of Waters (Milford, MA) PrePak RCM base packed with three 25 mm × 10 cm Prep Nova-Pak HR silica (particle size 6 μm) cartridges and a Guard-Pak insert, a U6K manual injector, a 510 pump, and a 481 LC spectrophotometer detector. A Baseline 810 Chromatography workstation (Waters) was used to record the chromatogram and calculate concentrations. The mobile phase was 4% (v/v.) ethyl acetate in hexane at a flow-rate of 21.6 mL / min. The fraction of γ-oryzanol was monitored at 330 nm which is the $\lambda._{max}$ for γ-oryzanol. The fraction of γ-oryzanol was collected and dried under nitrogen flow. High purity γ-oryzanol was prepared after drying.

3.1.5. Separation of individual components of γ-oryzanol in an analytical reverse phase HPLC

The analytical HPLC system that was used consisted of a Dynatech (Baton Rouge, LA) autosampler LC-241, a Waters 510 pump, a Hewlett-Packard (San Fernando, CA) UV-VIS diode-array detector (Series 1050), and a 25 cm × 4.6 mm diameter column of Microsorb-MV C_{18} (Rainin Instrument Company, Woburn, MA). The detector was set at 330 nm and 450 nm as reference. The mobile phase consisted of methanol, acetonitrile, dichloromethane, and acetic acid (50:44:3:3, by vol.) and the flow-rate was 1.4 mL / min.

Each peak was collected in a 15-mL test tube using a Gilson 202 fraction collector (Beltline-Middleton, WI) and absorbence (330 nm) was measured using a Gilford Response UV-VIS spectrophotometer (Okerlin, OH). Mobile phase was evaporated under nitrogen flow. Each component standard was obtained after drying. These components were used for TMS derivatization and GC-MS analysis for identification and quantification.

The concentration of γ-oryzanol was the sum of concentration of each individual component of γ-orzyanol.

3.1.6. Hydrolysis and derivatization

The component obtained from the collected peak was hydrolyzed with 1 mL of 0.1 N sodium hydroxide in methanol at 80°C (water bath) for 30 min. Ultra-high-purity nitrogen was flushed through the test tube for 15 sec during the addition of alkali. Then the test tube was tightly capped as quickly as possible. One milliliter of 0.1 N hydrochloride and 2 mL ethyl acetate were added to the solution after hydrolysis. The

solution was centrifuged at 1000×g. Then the upper organic layer was transferred to a 10-mL test tube containing sodium sulfate anhydrous. The lower layer was mixed with 2 mL ethyl acetate and extracted again. The upper organic layer was combined with the previous collection in a 10-mL test tube after the centrifugation. The sodium sulfate anhydrous was removed by filtering and the organic solvent was evaporated under nitrogen flow. The trimethylsilyl (TMS) ether derivative of the component was obtained by adding to the test tube 0.1 mL pyridine and 0.1 mL bis(trimethylsily)trifluoroacetamide (BSTFA) (Supelco Inc., Bellefonte, PA) that contained 1% trimethylchlorosilane. The reaction was performed at 30°C (water bath) for 30 min.

3.1.7. Gas chromatography (GC) and mass spectrometry (MS)

Derivatized components were determined using a SPB-5 fused silica column (Supelco Inc., Bellefonte, PA) that was 30 m length × 0.25 mm i.d. × 0.1 μm film thickness and a Hewlett-Packard 5790A GC coupled with 5970B mass selective detector (MSD). Helium was used as the carrier gas and maintained at a flow rate of 1.5 mL / min. Injection was splitless and injection temperature was 250°C. Oven temperature was programmed from 150 to 280°C at a ramp rate of 5°C / min. The initial and final hold times were 0 and 9 min, respectively. MSD conditions were as follows: capillary direct MS interface temperature, 280°C; ion source temperature, 280°C; ionization voltage, 70eV; mass range, 30 - 550 *m/z*; scan rate, 1.67 scans / sec; and electron multiplier voltage, 1800 V.

3.2. Solvent and supercritical fluid extraction of γ-oryzanol

3.2.1. Chemical and materials

All solvents were HPLC grade. Hexane was from Curtins Matheson Scientific Inc. (Houston, TX). Ethyl acetate was from EM Science (Gibbstown, NJ). Methanol, isopropanol, and dichloromethane were from Mallinckrodt Baker Inc. (Paris, KY). Acetonitrile and acetic acid were from Fisher Scientific Inc. (Fair Lawn, NJ). Ascorbic acid, sodium hydroxide, and sodium sulfate anhydrous were from Sigma (St. Louis, MO). Carbon dioxide used in supercritical fluid extraction was from BOC Gases (Riverton, NJ). Rice bran was a gift of the Riviana Rice Mill (Abbeville, LA).

3.2.2. Solvent extraction using hexane with saponification

One gram of rice bran was suspended in 5 mL distilled water in a 25-mL test tube. Two-tenth gram of ascorbic acid and 0.1 mL of 80% (w/v) sodium hydroxide were added to the test tube. The mixture was vortexed and incubated in a 60°C water bath for 30 min. The test tube was centrifuged 200×g for 15 min after 5 mL hexane was added and vortexed. The organic layer was collected in a funnel. The residual was mixed with 5 mL hexane and centrifuged again. The organic layer was combined with the previous collection. Distilled water was added to the funnel to rinse the organic layer. After the funnel was allowed to stand for 10 min, the distilled water was drained. The rinse step was repeated twice. Then, the organic layer was transferred to a conical tube. The extract was obtained after the organic solvent was completely evaporated under nitrogen flow.

3.2.3. Solvent extraction using hexane without saponification

One gram of rice bran was suspended in 5 mL distilled water in a 25-mL test tube. Two-tenths gram of ascorbic acid were added to the test tube. The mixture was vortexed and incubated in a 60°C water bath for 30 min. Five mL hexane was added to the test tube. The test tube was centrifuged at 200×g for 15 min. The organic layer was transferred to a conical tube. The residual was mixed with 5 mL hexane and centrifuged again. The two organic layers were combined and evaporated. The extract was obtained after the organic solvent was completely evaporated under nitrogen flow.

3.2.4. Solvent extraction using various solvents

The composition of solvents in extraction is listed in Table 2. One gram of rice bran and 5 mL of each solvent was added to a 25-mL test tube. The mixture was vortexed and incubated in a water bath of defined temperature. Then the test tube was centrifuged at 200×g. The suspension was collected in a conical tube. The residue was mixed with 5 mL of the same solvent or solvent mixture and centrifuged again. The two suspensions were then combined. Solvent in suspension was completely evaporated under nitrogen flow to obtain the extract. This process was replicated three times.

3.2.5. Supercritical fluid extraction

Supercritical fluid extraction was performed using a Dionex SFE-703 supercritical fluid extractor (Dionex Corporation, Sunnyvale, CA). Carbon dioxide (Grade 5.5) was used as a supercritical fluid. Figure 2 shows the typical device used in supercritical fluid extraction. The pressure of carbon dioxide was maintained at 680

atm during extraction. The temperature of each extraction was 30, 40, 45, 50, 55, 60, and 75 °C, respectively. Seven gram of rice bran was weighed and packed in the extraction cell of the supercritical fluid extractor. Five mL hexane was added to a collection vial. After collection, hexane was evaporated under nitrogen flow to obtain extract.

Table 2. Composition of tested solvent in solvent extraction (unit: percentage of volume)

No. of Solvent	1	2	3	4	5	6	7	8	9
Hexane	100	75	50	25	0	75	50	25	0
Ethyl Acetate	0	25	50	75	100	0	0	0	0
Isopropanol	0	0	0	0	0	25	50	75	100

3.2.6. Quantification for γ-oryzanol

γ-Oryzanol was quantified using a reverse-phase HPLC method. A C18 column (Rainin Instrument Company, Woburn, MA), a Waters 510 pump (Waters, Milford, MA), a Dyntech autosampler (Baton Rouge, LA), and a diode-array UV-VIS detector (Hewlett-Packard, San Fernando, CA), were employed in the HPLC system. The mobile phase consisted of methanol, acetonitrile, dichloromethane, and acetic acid (50 : 44 : 3 : 3). Flow rate was controlled at 1.4 mL /min and components were detected at 330 nm. Maxima chromatography workstation (Waters, Milford, MA) was used as signal recorder and to calculate each concentrations of individual component of γ-

oryzanol. The concentration of γ-oryzanol was obtained by summing all individual components.

3.2.7. Isolation and purification of major components of γ-oryzanol using a preparative scale reverse phase column

A Waters (Milford, MA) Delta Prep 400 HPLC system was modified in order to load precise amounts in any sample size for both small and large scale columns (Figure 3). A PrePak RCM base packed with two 25 mm × 10 cm Prep Nova-Pak HR C18 (particle size 6 μm) cartridges and a Guard-Pak insert was used as a preparative scale column to isolate and purify the three major components, cycloartenyl ferulate, 24-methylene cycloartanly ferulate, and campesteryl ferulate of γ-oryzanol. The wavelength of the Waters 470 UV detector was 330 nm. Signal of detector was recorded using a Maxima chromatography workstation (Waters, Milford, MA). The isolated components were collected using a Waters fraction collector (Milford, MA). The A, B, C, and D tubing for delivering mobile phase in Prep Delta 400 HPLC system were assigned to methanol, acetonitrile, dichloromethane, and acetate acid, respectively. The ratio of mobile phase was 50% A, 44% B, 3% C, and 3%D. The flow rate of mobile phase was 18 mL / min.

3.2.8. Statistical analysis

Replication for each solvent extraction was three times and for each supercritical fluid extraction was six times. The experimental data were analyzed using the General Linear Model procedure of the Statistical Analysis System (SAS, 1994). Significant difference between means was considered at $P<0.05$.

3.3. Quantitative analysis of antioxidant activity using a linoleic acid model

3.3.1. Chemical and materials

All solvents were HPLC grade. Hexane was from Curtin Matheson Scientific Inc. (Houston, TX). Isopropanol was from Mallinckrodt Baker Inc. (Paris, KY). Ethyl ether and acetic acid were from Fisher Scientific Inc. (Fair Lawn, NJ). α-Tocopherol, ferulic acid, and linoleic acid were from Sigma Company (St. Louis, MO).

3.3.2. Accelerated oxidation processing in linoleic acid

α-Tocopherol, ferulic acid, and the three high purity components of γ-orzyanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, obtained from the preparative scale reverse phase HPLC (described above) were prepared at concentrations of 0.08, 0.16, and 0.40 mM in hexane as treatment solutions. A pure hexane solvent was used as control. Linoleic acid solution (40 mM) was prepared by dissolving 1.1202 g of linoleic acid in 100 mL of hexane. Then, 5 mL of the linoleic acid solution and 5 mL of each treatment or control solution were mixed in a 25-mL test tube. The molar ratio of treatment to linoleic acid was 1:100 (0.40 mM treatment solution), 1:250 (0.16 mM treatment solution), and 1:500 (0.08 mM treatment solution). An aliquot of 500 μL of the mixing solution was transferred to a HPLC vial to determine initial concentrations of hydroperoxides. Then, the test tube was incubated in a 37°C water bath. Tubing connected to compressed air (BOC Gases, Port Allen, LA) was emerged in the test tube with the end of the tubing touching the bottom of test tube. The apparatus of accelerated oxidation processing is shown in Figure 4. The flow rate of air was controlled at 0.2 mL/min. Time of sampling was scheduled at 40, 80,

120, 160, and 200 min from the beginning of incubation. In the sampling step, 500 μL was taken from the solution and added to a HPLC vial after vortexing the solution well.

3.3.3. Analysis of hydroperoxides of linoleic acid

The hydroperoxides of linoleic acid were determined using a normal phase HPLC method. The HPLC system consisted of a Waters (Milford, MA) 510 pump, a 680 automated gradient controller, a 715 ultra sample processor, a Hewlett Packard (San Fernando, CA) UV-VIS diode-array detector, and a Baseline 810 chromatography workstation (Waters, Milford, MA), and Zorbax SIL (Du-Pont Co., Wilmington, DE) column. The mobile phase consisted of hexane, ethyl ether, isopropanol, acetic acid (100:15:0.1:0.1) and flow rate (min, mL / min) was 0-18, 1.8; 17-18, 1.8-2.0; 19-39, 2.0; 39-40, 2.0-1.8. Absorbance at 234 nm was monitored with the UV-VIS detector. The standards of hydroperoxides and hydroxides of linoleic acid, 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid, 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acid, 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid, 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic acid, 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid, 9-hydroxy-10-*trans*,12-*trans*-octadecadienoic acid, 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid, and 13-hydroxy-9-*trans*,11-*trans*-octadecadienoic acid were from Sigma (St. Louis, MO). Production of each hydroperoxide of linoleic acid at every sampling time was obtained by deducting initial concentration from final concentration.

Total production of hydroperoxides was obtained by summing all hydroperoxides. The rate (slope) of total production of hydroperoxides over time was used to evaluate the antioxidant activity for each tested compound.

3.3.4. Statistical analysis

Each treatment was evaluated in the linoleic acid model three times for antioxidant activity. The production of each hydroperoxide of linoleic acid was analyzed using the General Linear Model procedure of the Statistical Analysis System (SAS 1994). The rates of total production of hydroperoxides were analyzed using the Regression Model of the Statistical Analysis System (SAS 1994). The significant difference between rates for two compounds was considered at $P < 0.05$.

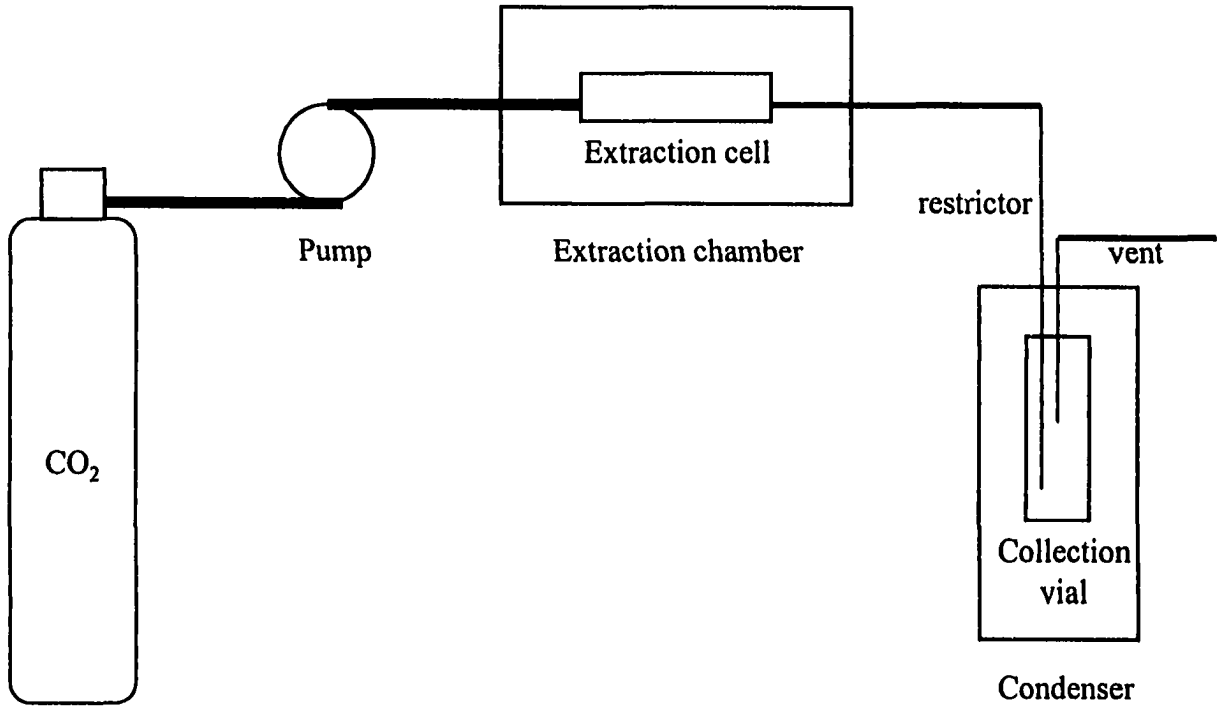
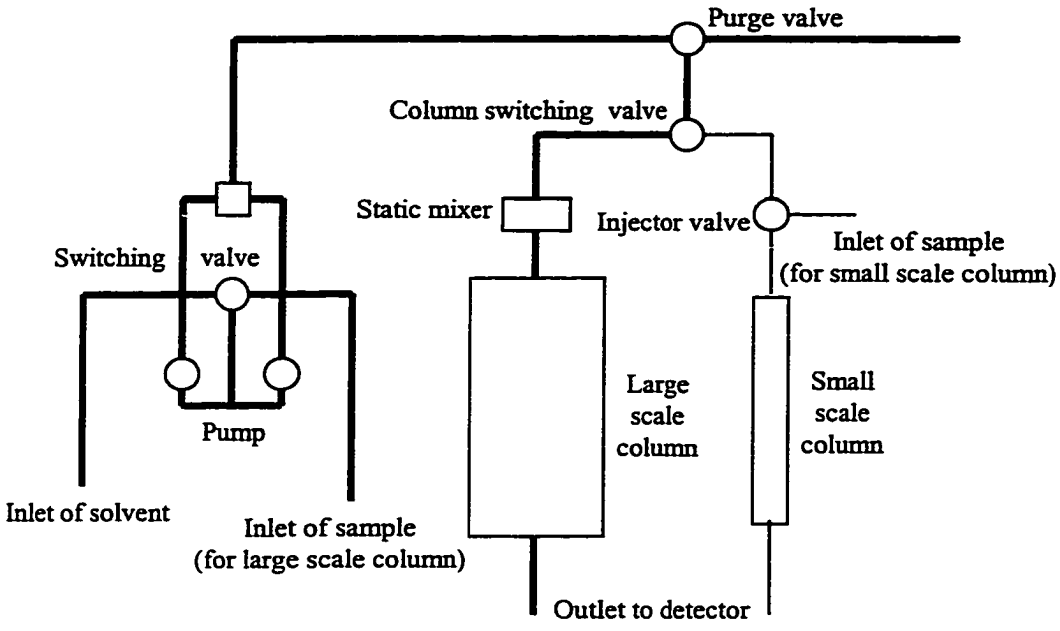
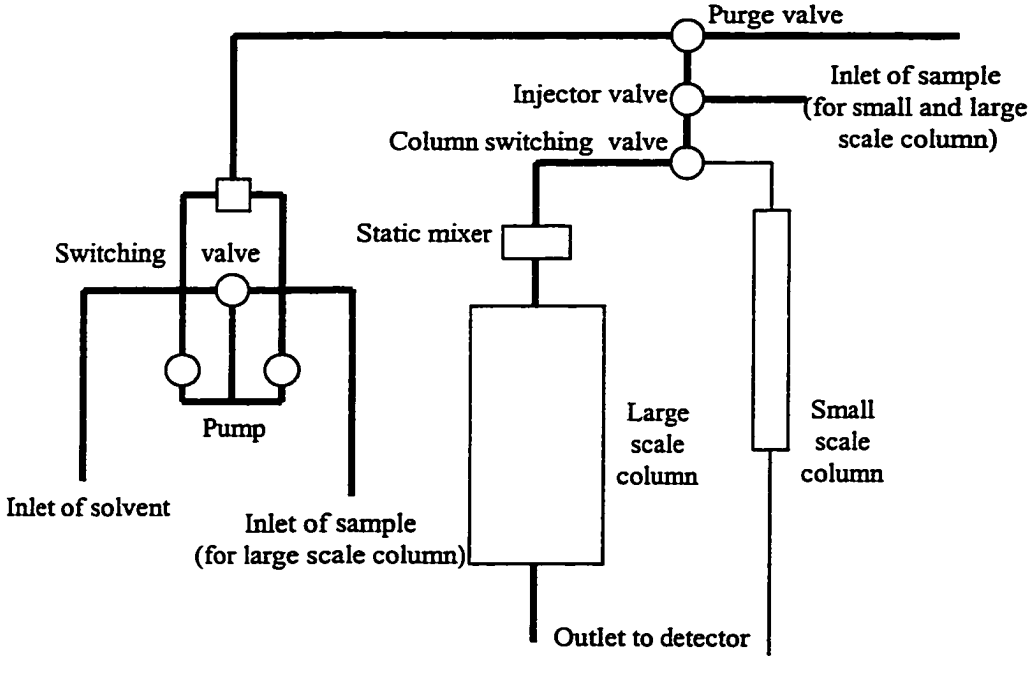


Figure 2. Device of supercritical fluid extraction.



(a) Before



(b) After

Figure 3. Waters preparative HPLC (Delta Prep 400) fluid pathway before and after modification.

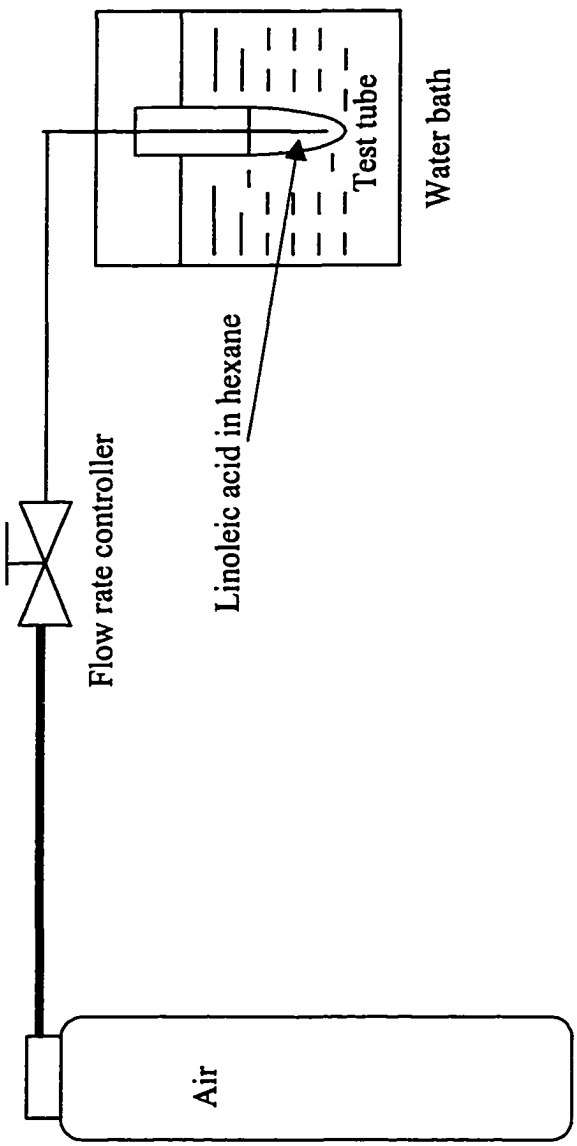


Figure 4. Apparatus of accelerated oxidation processing

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Separation and identification of components of γ-oryzanol

4.1.1. Extraction of crude oil

Crude oil of rice bran was extracted using a solvent extraction method without saponifcation. Saponification has usually been employed in most lipid extractions from plant and animal tissues. This procedure could remove interfering triglycerides and other hydrolyzable materials and aid to release lipid from sample matrix. A saturated aqueous solution of Na_2CO_3 or KOH was used in rice bran oil extraction by Norton (1995) and Diack and Saska (1994), respectively. However, saponification may also hydrolyze the ester bond between triterpenoids and ferulic acid components of γ-oryzanol to produce ferulic acid and triterpenoids. The effect of saponification on extraction of γ-oryzanol will be reported in the next section of this chapter. The yield of γ-oryzanol in extract obtained using hexane was lower than that that using a mixture of hexane and ethyl acetate (7:3, v/v). This ratio of hexane and ethyl acetate produced a higher yield of γ-oryzanol, compared to hexane, or other ratios of hexane and ethyl acetate. Details of the effect of solvent polarity on the yield of γ-oryzanol are also reported in the next section of this chapter. Ascorbic acid was added during extraction in order to prevent the oxidation reaction from occurring. Three and half gram of crude oil was obtained from 25 g of rice bran under this extraction condition.

4.1.2. Semi-purification of γ-oryzanol using a low pressure silica column

Figure 5 is a chromatogram of crude rice bran oil flushed by different ratios of hexane and ethyl acetate in a low pressure silica column. The amount loaded on the column was 3.5 g crude oil in 10 mL of hexane. Most of the low polarity lipid was flushed out of the column with 50 mL of the solvent mixture hexane : ethyl acetate (9:1, v/v). γ-Oryzanol was eluted when the ratio of the mixture was changed to 7:3 (hexane : ethyl acetate, v/v). This eluent was collected and dried under nitrogen flow from which seven-tenths gram of semi-purified γ-oryzanol was obtained.

4.1.3. Purification of γ-oryzanol using a preparative scale normal phase HPLC

Five commercial silicas were compared for their resolution in separating the individual components of γ-oryzanol from rice bran oil (Diack and Saska, 1994). The Nova-Pak (Waters) silica was found to possess the most desirable attributes of the five silicas. In this study, three 25 mm × 10 cm Prep Nova-Pak HR silica cartridges and a Guard-Pak insert were used as a preparative scale normal phase column to purify γ-oryzanol from semi-purified γ-oryzanol. The semi-purified γ-oryzanol obtained from the low pressure chromatography was diluted with dichloromethane and injected on the preparative HPLC column. The chromatogram of semi-purified γ-oryzanol in the normal phase preparative scale HPLC is shown in Figure 6. The γ-oryzanol peak was eluted at 35 to 42 min and collected. The profile of the chromatogram was the same as that of Diack and Saska (1994) in analytical normal phase HPLC. There were several fractions occurring before γ-oryzanol eluted from the column, since semi-purified γ-

oryzanol contained a relatively high amount of interfering lipids, such as triglycerides, tocopherols, and tocotrienols.

The solvent of the γ-oryzanol fraction was evaporated, yielding approximately 36 mg of purified γ-oryzanol from the 0.7 g of semi-purified γ-oryzanol.

4.1.4. Separation of individual components of γ-oryzanol in an analytical reverse phase HPLC

Figures 7 and 8 are the chromatograms of γ-oryzanol components in an analytical reverse phase HPLC after injecting high purity γ-oryzanol and crude rice bran oil obtained directly from solvent extraction, respectively. The numbered peaks in Figure 7 were collected for identification of their structures using GC-MS. Components of γ-oryzanol in rice bran oil were separated using reverse-phase HPLC methods by Evershed et al. (1988), Norton (1994 and 1995), Rogers et al. (1993), and Seitz (1989). However, the resolutions of these separations were not entirely satisfactory and some peaks had not been identified. Acetonitrile / *n*-butanol / acetic acid / water (94:3:2:1, v/v.) was used as the mobile phase in Norton (1995), which produced the highest resolution of separation among these methods. In this study, the mixture of methanol / acetonitrile / dichloromethane / acetic acid (50:44:3:3, v/v) was used as the mobile phase with a run time of 42 min. The resolution of separation was visibly improved with a greater number of separated peaks. The components in peak 3, 4, and 6 in Figure 8 were major components of γ-oryzanol from rice bran. This was similar to the results of Norton (1995), Rogers et al. (1993), and Evershed et al. (1988). However, some minor components that were not found in these studies, such as peak 1, 2, 5, 7, 8, 9, and

10, were observed in this study. Although a large amount of high purity γ-oryzanol was loaded on the analytical column, with responses that were out of range for the detector, peaks of non-γ-oryzanol components were much smaller than that found with the loading of a small amount of crude rice bran oil (Figure 7 and 8). This indicated that most interfering lipids of rice bran oil were removed efficiently after purification using low pressure and preparative scale normal phase chromatography.

4.1.5. Absorption spectrums of components of γ-oryzanol using a spectrophotometer

The spectrum of absorbency of one component of γ-oryzanol, sitostanly ferulate, is shown in Figure 9. All of the individual components had similar absorption curves with highest absorbance at a wavelength of 330 nm. This result is comparable to that of Diack and Saska (1994).

4.1.6. Mass spectras of components of γ-oryznaol using GC-MS

The total ion chromatogram (TIC) of gas chromatograph-mass spectrometry (GC/MS) of an individual TMS derivative, sitostanol-TMS ether, is shown in Figure 10 as an example. Sitostanol-TMS ether showed a single major peak in the chromatogram. Peaks having retention time less than 15 min in the GC chromatogram were by-products of derivatization, such as trifluoroacetamide, trimethylsilyltrifluoroacetamide, and ferulate-TMS ether. The retention times of TMS derivatives of components of γ-oryzanol varied from 24 to 28 min in each GC chromatogram.

Table 3 lists molecular ion and major fragment ions in mass spectra of each individual TMS derivative. The structures that were constructed based on the information from mass spectras are depicted in Figure 11(a) and 11(b). A fair number

of useful fragment ions were used to interpret structure of components produced in electron ionization. In low mass range of every mass spectrum, there were *m/z* 73 and 75 ions, which had relatively high abundance peak. The two ions were explained as $((CH_3)_3Si)^+$ and $(HO{=}Si(CH_3)_2)^+$, respectively. Also, the presence of an ion at *m/z* 147 was $((CH_3)_2Si{=}O\text{-}Si\ (CH_3)_3)^+$ formed from two or more TMS groups in a molecule (Evershed, 1993). The molecular ion of each individual TMS derivative appeared in this study, even though some were of low intensity. The $(M\text{-}15)^+$ was not a major fragment ion. However, $(M - TMS)^+$ and $(M - TMS - CH_3)^+$ were obtained as relatively high abundance ions in each mass spectra. For cycloartenol-TMS and 24-methylene cycloartanol-TMS, $(M - TMS - CH_3 - CH_3 - CH_2 + H)^+$ was an important major fragment ion that did not appear in Δ^7-stenol-TMS, sterol-TMS, and stanol-TMS. It may have occurred because methyl groups in the structure of the triterpene of cycloartenol-TMS and 24-methylene cycloartanol-TMS were readily taken from the molecule during ionization. Many major fragment ions of stanol-TMS in mass spectra were larger than that of Δ^7-stenol-TMS or sterol-TMS by two units of *m/z*. The reason for this is that there is no double bond in triterpene structure of stanol-TMS, which does occur in the structure of Δ^7-stenol-TMS and sterol-TMS. The most difficult aspect of identification for these components was in distinguishing Δ^7-stenol-TMS and sterol-TMS since they have the same molecular weight and similar structure. The differences in mass spectra between them in this study were the *m/z* 129 that was a moderate ion in Δ^7-stenol-TMS but one of the highest ions in sterol-TMS. In contrast, the *m/z* 229 that was of lower abundance in sterol-TMS was a higher abundance ion in Δ^7-stenol-TMS.

Table 3. Molecular masses and major fragmentation ion of TMS ether derivatives of each peak collected in reverse-phase HPLC

Peak	Molecular ion (*m/z*)	Major Fragment ions (*m/z*)	Identification
1	484	379, 255, 229, 213, 173, 159, 145, 143, 129, 119, 105, 93, 73	Δ^7-Stigmastenol-TMS
2	484	394, 379, 355, 255, 213, 173, 159, 45, 143, 129, 119, 105, 95, 73	Stigmasterol-TMS
3	498	408, 393, 365, 339, 297, 255, 203, 187, 159, 149, 131, 95, 81, 69	Cycloartenol-TMS
4	512	422, 407, 379, 297, 203, 187, 175, 173, 159, 135, 95, 73, 69	24-Methylene Cycloartanol-TMS
5	472	382, 367, 255, 229, 213, 173, 159, 145, 129, 121, 107, 95, 85, 75	Δ^7-Campestenol-TMS
6	472	382, 367, 234, 289, 261, 255, 213, 173, 159, 129, 121, 107, 95, 85, 75	Campesterol-TMS
7	486	471, 396, 381, 255, 229, 213, 173, 159, 145, 129, 121, 105, 95, 75	Δ^7-Sitostenol-TMS
8	486	396, 381, 357, 275, 255, 213, 173, 159, 145, 129, 121, 105, 95, 75	Sitosterol-TMS
9	474	384, 369, 257, 215, 173, 161, 147, 129, 121, 107, 95, 85, 75	Campestanol-TMS
10	488	473, 398, 383, 359, 305, 257, 215, 173, 161, 147, 129, 121, 105, 95,75	Sitostanol-TMS

The relatively high intensity *m/z* 229 ion was a characteristic of Δ^7-stenol compound (Gustafsson et al., 1966). Figure 12 shows the most probable mechanism of formation of *m/z* 129 and *m/z* 229 in the sterol-TMS and Δ^7-stenol-TMS, respectively. For the sterol-TMS, carbon 1, 2, and 3 together with TMS were readily lost during

ionization and formed the *m/z* 129 fragment ion. For the stenol-TMS, the loss of the TMS group, the carbon 16 and 17, and the side chain of carbon 17 often occurs at the same time. The molecular weight of the residual ion was *m/z* 229.

4.1.7. Components of γ-oryzanol

From mass spectras of TMS derivatives and absorbence curves of components of γ-oryzanol, ten components were positively identified in this study. They are listed in Table 3 according to the order of retention time in the chromatogram of crude rice bran oil in the analytical reverse phase HPLC (Figure 8). The molecular size of each component of γ-oryzanol is very close. Therefore, the molecular size was not an important factor that affected the retention time of each component in chromatography. It is suggested that the peak retention times of components of γ-oryzanol in the analytical reverse phase HPLC are largely dependent on the number and positions of double bonds, since they are related to the polarity of compounds, although the relationship is not strong. Evidence was found that the retention time of components containing two double bonds was less than that of components having one double bond or no double bond. Components having a double bond on a side chain of the triterpene eluted prior to the components having a double bond in the triterpene.

The three largest peaks were cycloartenyl ferulate (peak 3), 24-methylene cycloartanyl ferulate (peak 4), and campesteryl ferulate (peak 6). These are the major components of γ-oryzanol in rice bran oil. This result was consistent with other studies (Evershed et al., 1988; Norton, 1995; Rogers et al., 1993). Compared to the three major components in rice bran oil, the peak areas of other components were much smaller.

This situation usually is a limitation in implementing separation conditions for all components of γ-oryzanol since minor components are readily neglected when they are eluted with a major component. Peaks 8 and 9 that were identified as sitosteryl ferulate and campestanyl ferulate, respectively, were not separated as two individual peaks in Evershed et al. (1988) and Rogers et al. (1993). They occurred as a single peak in their chromatograms and were considered as sitosteryl ferulate (Evershed et al., 1988) or a mixture of sitosteryl ferulate and cycloartanyl ferulate (Rogers et al., 1993). Although they were separated in Norton (1995), one was identified as sitostanyl ferulate, which differs from this study and the other was considered an unknown. Stigmasteryl ferulate was reported to elute with campesteryl ferulate in a single peak (Rogers et al., 1993). However, they were successfully separated as two individual peaks in this study. Stigmasteryl ferulate was peak 2, with a shorter retention time. This supports the contention that a component with more double bonds in the triterpene portion or in its side chain has shorter retention time in reverse phase HPLC. The last peak in our chromatogram was identified as sitostanyl ferulate. This was an unknown peak in Rogers et al. (1993) and identified as cycloartanyl ferulate in Norton (1995).

In this study, four of the ten identified components, Δ^7-Stigmastenyl ferulate, Δ^7-campestenyl ferulate, Δ^7-sitostenyl ferulate, and campestanyl ferulate, which were not reported to be in rice bran oil previously, were separated and identified. They had much smaller peak areas. The successful separation in this study has contributed to the purification of γ-oryzanol from crude rice bran oil using low pressure chromatography

and preparative scale normal phase HPLC. After γ-oryzanol was highly purified, a highly concentrated γ-oryzanol could be injected in a relatively large amount on the analytical column without losing resolution. This is beneficial in implementing separation because minor components produced relatively high detector responses under these conditions.

4.2. Solvent and supercritical fluid extraction of γ-oryzanol

4.2.1. Solvent extraction of γ-oryzanol using hexane with and without saponification

A significant difference between the concentrations of γ-oryzanol in extracted oil using hexane with and without saponification is shown in Table 4. The concentration of γ-oryzanol in extracted oil without saponificaton was about two times higher than that with saponification. The structure of γ-oryzanol may be decomposed during saponification because the ester bond between ferulic acid and the triterpene component of γ-oryzanol was hydrolyzed under alkali condition (Figure 13). Although the advantage of saponification in many lipid extractions is breaking down the matrix of sample to free compounds of interest and reducing interfering lipids, it has a significant reverse effect on the yield of γ-oryzanol in extraction. Norton (1995) reported that 3.4 mg γ-oryzanol per gram of rice bran oil was extracted when saponificatoin was performed using a saturated aqueous solution of Na_2CO_3. Roger et al. (1993) also reported that 0.115 to 0.787 mg γ-oryzanol per gram could be extracted from refined rice bran oils that were pretreated using alkali solutions. These concentrations were lower than that without saponification in this study.

Table 4. Yield of extracted oil and concentration of γ-oryzanol in the extracted oil from 1 g rice bran using hexane with and without saponification

	Weight of oil extract (g)	Conc. of γ-oryzanol in oil extract (mg/g)
With saponification	0.14 ± 0.01	4.6 ± 0.5
Without saponification	0.15 ± 0.01	9.8 ± 0.2

4.2.2. Solvent extraction of γ-oryzanol using different solvent ratios at different temperatures

The weights of extracted oil obtained using different solvent mixtures were very close and approximately 0.13 to 0.15 g per gram of rice bran. However, there were significant differences between concentrations of γ-oryzanol in these extracted oils. Figure 14 shows the concentrations of γ-oryzanol in extracted oils obtained using different solvent ratios. There were three groups that had significantly higher concentrations. High concentrations of γ-oryzanol could be extracted from rice bran with solvent mixtures of hexane : isopropanol (50:50, v/v) at 45°C, hexane : isopropanol (75:25, v/v) at 60°C and hexane : isopropanol (50:50, v/v) at 60°C. Lower concentrations of γ-oryzanol were extracted with hexane : isopropanol (25:75, v/v) at 30°C and hexane: isopropanol (0:100, v/v) at 30°C. The other solvent mixtures at different temperatures extracted intermediate concentration of γ-oryzanol. Figure 14 also shows a trend towards a positive relationship between concentration of γ-oryzanol in extracted oil and extraction temperature. This may be related to the physical property of the matrix of the sample, which is more penetrable at higher temperatures and causes γ-oryzanol to be released from the matrix. Temperature of extraction was an especially important factor affecting concentration of γ-oryzanol when isopropanol was used.

Both highest and lowest concentrations of γ-oryzanol in extracted oil were obtained when using solvents containing isopropanol. For solvents containing ethyl acetate, the effect of temperature on concentration of γ-oryzanol was not significant. The solvents containing ethyl acetate or only hexane readily penetrated the rice bran matrix to extract γ-oryzanol regardless of the physical property of sample matrix as affected by temperature. This may be due to the fact that these solvents had lower polarity and preferentially extract lipids. A lower affinity for non-polar lipid and lower penetration capability of isopropanol compared to hexane or ethyl acetate may be due to it is higher polarity and viscosity. Therefore, the extraction using solvents containing a high percentage of isopropanol depended on high temperatures to assist in loosening the structure of the sample matrix and increase mobility of isopropanol. When the solvent contained isopropanol and had greater access to lipids, there was greater capacity to extract γ-oryzanol than solvents containing ethyl acetate or only hexane. However, temperatures above 60°C in solvent extraction have disadvantages in practice, such as evaporation of extraction solvent.

4.2.3. Concentration of γ-oryzanol in extracted oil at different extraction times using hexane : isopropanol (50:50, v/v) at 60°C

Concentrations at 30, 45, and 60 min were 11.0, 11.6, and 11.7 mg γ-oryzanol per gram of extracted oil, respectively (Figure 15) when using hexane : isopropanol (50:50, v/v) at 60°C. Significant differences occurred between 30 vs. 45 min and 30 vs. 60 min. However, the difference in concentration between 45 and 60 min was not significant. Therefore, the extraction time could be shortened to 45 min without significantly affecting the concentration of γ-oryzanol in extracted oil.

4.2.4. Yield of extract using supercritical fluid extraction (SFE) under different conditions

Figure 16 shows the yield of extract of SFE at different extraction times and various temperatures. The yields of extract in 10 min extraction time were significantly increased at 55, 60, and 75°C. For extraction temperatures above 55°C, yields were the highest and changed slightly after 10 min extraction. For extraction temperatures of 30, 40 and 45°C, yields continued to increase over time. The effect of higher extraction temperature in SFE may be similar to that in solvent extraction where it may alter the physical property of the sample matrix and make it more penetrable by extraction fluid. Therefore, high extraction temperature in SFE resulted in higher yield at shorter extraction times.

The range of yields of extracts in 20 min extraction time was 0.64 g at 30°C to 0.92 g at 60°C from 7 g of rice bran. The percentage yield of extract from rice bran was 9.1 to 13.1 %. Yields were lower than that of solvent extraction (approximately 14%). Garcia et al. (1996) reported that the highest yields for SFE were obtained at a pressure of 28 MPa (280 atm) and a temperature of 70°C for four hours, but total yield was only 7.1 %. Also, they concluded that the yield of γ-oryzanol could be improved with more severe extraction conditions. In this study, however, the yield of extract at higher extraction temperature (75°C) was not significantly higher than that of 55 and 60°C. Tsuda et al. (1995) found that the yield of extracts at 40°C also was higher than that of 60 and 80°C. It is suggested that severe SFE conditions may not assist to increase yield of extract. It is possible that more extract escaped from the vent of the SFE system because it was not condensed in the collection vial due to high extraction temperature

and pressure. Therefore, yield of extract was not positively associated with extraction temperature. However, long extraction times with lower extraction temperatures also is not good practice since it results in high consumption of expensive supercritical carbon dioxide.

4.2.5. Yields and concentrations of γ-oryzanol in extracts using SFE at different times and temperatures

Figure 17 shows the yields of γ-oryzanol in extracts at different extraction time and temperature using SFE. In 10 min extraction, the increase of γ-oryzanol in extract was significantly higher at extraction temperatures of 55, 60, and 75°C compared to lower temperatures. After 10 min, however, γ-oryzanol in extract did not increase further at these extraction temperatures. Furthermore, the γ-oryzanol in extracts at extraction temperatures of 60 and 75°C for 20 min was significantly lower than that of extraction temperature of 50°C. In the first 10 min of extraction, a large amount of extractable material in rice bran was already extracted at these higher extraction temperatures (Figure 17). During 10 to 20 min extraction, a lesser amount of extractable material in rice bran was extracted at these temperatures. Because γ-oryzanol and other components were more mobile in the SFE system at lower concentration and higher extraction temperature, they did not condense completely in the collection vial and escaped from the vent. Thus, yields of γ-oryzanol did not increase further.

Table 5 lists yields and concentrations (above 100 mg per gram extract) of γ-oryzanol in extracts at different extraction times. The highest yield of γ-oryzanol in

SFE was in 15 - 20 min at 45°C extraction temperature and highest concentration in extract was at 15 -20 min at 50°C extraction temperature. From the results in Table 5, it is suggested that γ-oryzanol could be concentrated and extracted from the sample matrix under a period of optimum extraction conditions. This is an important consideration that may establish SFE as a valuable method in chemical extraction and separation. The high concentration of γ-oryzanol in extract obtained from SFE without the use of toxic organic solvents may be preferred in food and pharmaceutical industries. SFE conditions can be selected based on extraction either for high yield or high concentration of γ-oryzanol. However, limitations of the SFE device, such as fluctuation of flow rate and pressure that are important factors in extraction, caused relatively high standard deviations.

Table 5. Yields and concentration of γ-oryzanol from 7.0 g of rice bran in extract at different peroid of extract time and temperature

Temperature (°C)	Time (min)	Yield (mg)		Conc. (mg/g)	
40	15 - 20	9.3 ± 1.7	A	332.3 ±136.8	B,C
45	15 - 20	11.8 ± 0.8	A	227.7 ± 65.4	B,C
	20 - 25	7.7 ± 3.7	A	484.0 ± 88.1	A,B
50	15 - 20	7.8 ± 3.7	A	674.6 ±148.1	A
	20 - 25	0.9 ± 0.7	B	132.1 ± 81.8	C

Significant difference between groups without same letter.

4.2.6. Comparison of yields and concentrations of γ-oryzanol in extracts between SFE and solvent extraction

Table 6 lists the yields and concentrations of γ-oryzanol in extracts obtained from solvent extraction and SFE, respectively. The yield of SFE was three times higher than that of solvent extraction. Also, a large amount of solvent and time consumed in

solvent extraction were eliminated in the SFE method. Furthermore, a much higher concentration of γ-oryzanol was obtained in SFE when extract was collected during a specific period (15 - 20 min) of extraction time.

This process would allow higher loading on preparative scale columns for purification of individual components of γ-oryzanol and eliminate the need for low pressure chromatography to concentrate the γ-oryzanol (as described previously). The extract of SFE could be loaded on the preparative scale column directly without any purification step. A chromatogram of the extract of SFE in preparative scale reverse phase HPLC is shown in Figure 18. Compared to solvent extraction, SFE eliminated the use of expensive organic solvent in extraction, nitrogen for evaporating solvent, and the need for a low pressure chromatography clean up procedure, which were necessary in solvent extraction.

Table 6. Comparison of yields and concentrations of γ-oryzanol in extracts between SFE and solvent extraction

Item	Solvent extraction[1]	SFE[2]	SFE[3]
Yield (mg /g of rice bran)	1.68 ± 0.02	5 .39 ± 0.43	1.11 ± 0.07
Conc. (mg/ g of extract)	11.8 ±0.2	51.0 ± 5.5	674.6 ±148.1

[1]extraction with hexane : isopropanol (50:50) at 60°C for 60 min.
[2]extraction under 680 ATM at 50°C for 25 min.
[3]extraction under 680 ATM at 50°C and collection between 15 - 20 min.

4.3. Quantitative analysis of antioxidant activities of α-tocopherol, ferulic acid and the three major γ-oryzanol components using a linoleic acid model

4.3.1. Hydroperoxides and hydroxides of linoleic acid

In this study, four hydroperoxides and hydroxides of linoleic acid were separated using normal phase HPLC. Figure 19 shows chromatograms of

hydroperoxides and hydroxides of linoleic acid during oxidation. The mechanism of formation of hydroperoxides of linoleic acid is shown in Figure 20. Linoleic acid is much more susceptible to oxidation because its structure contains 1,4-pentadiene. The methylene group at position 11 is highly vulnerable during free radical attack (Nawar, 1996). Two conjugated dienes with free radicals, 10-,12- and 9-,11-, are formed after linoleic acid reacts with free radical. Generally, the new double bond that is formed has *trans* structure after geometric isomerization, which has lower energy than the *cis* structure (Nawar, 1996). Thus, 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid (9HPODE(t,c)), 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acid (9HPODE(t,t)), 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (13HPODE(c,t)), and 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic acid (13HPODE(t,t)) are produced as primary oxidation products of linoleic acid. Also, 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid (9HODE(t,c)), 9-hydroxy-10-*trans*,12-*trans*-octadecadienoic acid (9HODE(t,t)), 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid (13HODE(c,t)), and 13-hydroxy-9-*trans*,11-*trans*-octadecadienoic acid (13HODE(t,t)) are produced during the sequence of free radical chain reactions.

4.3.2. Developing quantitative analysis of hydroperoxides of linoleic acid

Currently, the methods that are used to analyze hydroperoxides involve monitoring the formation of conjugated diene at absorbance at 234 nm or by titrating iodine which is oxidized from I^- by hydroperoxides (Corongiu and Banni, 1994; Jessup et al., 1994). These methods lack sensitivity and specificity to give reliable oxidation status of lipids. To overcome these deficiencies, chromatography methods are being

developed to analyze hydroperoxides. Thomas et al. (1992) and Guido et al. (1993) utilized gas chromatography to perform this analysis. Although gas chromatography enhances the sensitivity and specificity of analysis, it has disadvantages due to complex derivatization of sample and decomposition of hydroperoxides during high temperature analyses. In HPLC methods, post-column chemiluminescence reactions were used to convert hydroperoxide to products that had high sensitivity for detectors since UV detection was considered to be of low sensitivity for lower concentrations of hydroperoxides (Miyazawa et al., 1994; Yamamoto et al., 1994). However, many factors related to the post-column reaction, such as temperature and pulse of flow rate, reduce the chances of obtaining satisfactory results. The low response of the UV detector in analyzing hydroperoxides may result not only from low concentrations of hydroperoxides in sample but also incomplete separation. In incomplete separation, each hydroperoxides may not be formed as a significant fraction at high enough concentration to obtain satisfactory detector responses. In this study, sufficient resolution of each hydroperoxide was obtained using an analytical normal phase HPLC column and UV detector after the separation conditions for hydroperoxides of linoleic acid were optimized (Figure 19). However, the fractions of hydroxides of linoleic acid were not as significant as that of hydroperoxides. The concentration of hydroxides may be much lower than that of hydroperoxides in oxidation of linoleic acid. Also, hydroxides are not as important as hydroperoxides as indicators of the degree of lipid oxidation. Therefore, hydroperoxides of linoleic acid were quantified in this study to evaluate oxidation status of linoleic acid.

The order of elution of the eight products separated using a normal phase HPLC column was 13HPODE(c,t), 13HPODE(t,t), 9HPODE(t,c), 9HPODE(t,t), 13HODE(c,t), 13HODE(t,t), 9HODE(t,c), and 9HODE(t,t). Since the structure and molecular weight of linoleic acid hydroperoxides and hydroxides are highly similar, it is difficult to separate them individually within a short retention time. The total running time was 40 min with gradient flow rate of mobile phase that contained hexane, diethyl ether, isopropanol, and acetic acid. Since the short wavelength (234 nm) used in the UV detector was very close to the UV cutoff wavelength of diethyl ether, isopropanol, and acetic acid, the baselines of chromatograms were slightly shifted and noisy.

4.3.3. Linoleic acid model

Lipid oxidation involves many environmental factors, such as light, oxygen, free radicals, and metal ions (Gordon, 1990; Frankel, 1991). Many model systems have been employed to investigate oxidation of lipids and antioxidant activities of compounds. Frankel (1991) reviewed and reported the limitation of accelerated stability models, which were performed by increasing temperature and oxygen concentration in a lipid system. Reliable results were obtained when at room temperature. However, a lower yield of oxidation products in mild conditions made quantitative determination within hours more difficult. Chemical system models to generate oxygen radicals have also been studied intensively. Sources for OH free radicals comprised Fenton systems in aqueous solutions (Puppo, 1992) or specific generation systems in organic solvents (Grant et al., 1984; Tezuka, 1988). However, these models were much more complex and more difficult to understand due to the

multitude of radicals formed (Greenley and Davies, 1992). Enzymatic generation models also had limitations in the investigation of antioxidant activity because of the restrictive structure of enzymes.

On the another hand, the thiobarbituric acid (TBA) test also is a widely used approach in monitoring oxidation of lipids. However, the test results were questionable due to many factors (Chirico, 1994). The chromogens that the test depends on are formed by various aldehydes, besides malondialdehyde. The heating step in the test results in further oxidation of sample and an overestimated value. Malondialdehyde is a secondary oxidation product that is not sensitive in evaluating the degree of oxidation in short time. Thus, the TBA test has low sensitivity and specificity in determination of early stages of oxidation.

Figure 19 gives the chromatograms of hydroperoxides and hydroxides of linoleic acid at 0, 120, and 200 min in the linoleic acid model used in this study. The four hydroperoxides increased significantly with increasing oxidation time under continuous input of air (0.2 mL/min) to the linoleic acid model. However, the changes of the four hydroxides were not as great. These results reflected the status of oxidation in early stages of lipid oxidation. As primary oxidation products, hydroperoxides were produced in large quantities by oxidation reactions during the early stage of oxidation. Their rate of formation exceeded their rate of decomposition. From Figure 19 and the control group of Table 7, the increasing amounts of 13HPODE(t,t) and 9HPODE(t,t) were significantly higher than that of 13HPODE(c,t) and 9HPODE(t,c) during oxidation with air. This imbalance or disproportional formation of hydroperoxide isomers

indicated that different formation mechanisms exist between HPODE (t,t) and HPODE (t,c) or HPODE (c,t). Hydroperoxide molecules containing *trans,trans* are more thermodynamically favorable to be formed than that having *trans,cis* or *cis, trans* during continuous air input. Nawar (1996) also reported that 9- and 13- *cis,trans* diene hydroperoxides undergo interconversion, along with some geometiric isomerization forming *trans,trans*- isomers. Therefore, the major hydroperoxides of linoleic acid formed after lipids are attacked by singlet oxygen were hydroperoxides containing *trans,trans* structure. Each hydroperoxide may also be produced by the free radical chain reaction involving radicals existing in the system prior to the input of air. The significant change of concentration for each hydroperoxide in the linoleic acid model using the HPLC method demonstrated that this system could be used in quantitative determination of the status of linoleic acid oxidation. It is very sensitive and fast since the determination can be completed in hours. Also, the mild conditions of oxidation in this model, 37°C and gentle input of air, resulted in more reliable experimental data than some other models that used severe or complex oxidative conditions (Frankel, 1991).

4.3.4. Profile of production of hydroperoxides in α-tocopherol treatment

Figure 21 shows the chromatograms of hydroperoxides and hydroxides of linoleic acid after adding α-tocopherol (α-tocopherol : linoleic acid molar ratio = 1:250) at 0, 120, and 200 min. Compared to the control, the concentration change of each hydroperoxide was slight (Table 7). Significant antioxidant activity of α-tocopherol was observed in this linoleic acid model. From Figure 21 and Table 7, the production of 13HPODE(c,t) and 9HPODE(t,c) was significantly higher than that of 13HPODE(t,t)

and 9HPODE(t,t) in the α-tocopherol treatment, even though all were much lower than the control. The profile of production of these hydroperoxides in the α-tocopherol treatment was contrasted to changes in the control. α-Tocopherol had high antioxidant activity against oxidation due to air. This indicated that the singlet oxygen, which is a major contributor to the formation of hydroperoxides containing *trans,trans* structure, was reduced largely after adding α-tocopherol to the linoleic acid model. Also, the free radical chain reaction caused by radicals initially existing in the system and producing hydroperoxides was mostly inhibited in view of slight production of hydroperoxides containing *trans,cis* or *cis,trans* structure. Two primary antioxidation mechanisms for α-tocopherol, chain-breaking electron donor and chain-breaking acceptor mechanism were discussed in Briviba and Sies (1994). In the chain-breaking electron donor mechanism, α-tocopherol reacts with peroxy radical by readily transferring a hydrogen atom to the peroxy radical to produce the more stable hydroperoxide. The chain-breaking acceptor mechanism includes singlet oxygen scavenging. Clough et al. (1979) reported that singlet oxygen scavenging is more predominant and hydroperoxydienone tocopherol is formed after α-tocopherol quenched a singlet oxygen. The results in this study supported their explanations and α-tocopherol appears to have a strong ability in scavenging singlet oxygen and free radicals, such as peroxyl radical.

4.3.5. Profile of production of hydroperoxides in ferulic acid treatment

Figure 22 shows the chromatograms of hydroperoxides and hydroxides of linoleic acid with ferulic acid (ferulic acid : linoleic acid molar ratio = 1:250) at different oxidation times (0, 120, and 200 min). The production of hydroperoxides in

ferulic acid treatment are listed in Table 7. Antioxidant activity of ferulic acid was observed through lower concentrations of each hydroperoxide, compared to the control group. Different profiles for production of the hydroperoxides were observed between ferulic acid and α-tocopherol treatment. The production of hydroperoxides containing *trans,trans* structure in the ferulic acid group was significantly higher than that in the α-tocopherol group. However, the production of hydroperoxides containing *trans,cis* or *cis,trans* was lower in ferulic acid treatment than α-tocopherol treatment. Therefore, the mechanism of antioxidation of ferulic acid was not the same as that of α-tocopherol. The antioxidant activity of ferulic acid appeared mostly in reducing free radical reactions due to radicals, such as peroxy radical, which initially existed in the system or were produced during oxidation. The ability of ferulic acid to scavenge singlet oxygen was not as high as α-tocopherol. Ferulic acid, as a type of phenolic acid, has been reported to have antioxidant activity (Marinova and Yanishieva, 1994). Generally, phenolic antioxidants inhibit lipid oxidation by trapping the peroxy radical to yield the hydroperoxide. They prevent the reaction of lipid and peroxy radical to produce the lipid radical and propagate free radical chain reactions. The results obtained for ferulic acid treatment support this explanation. Also, the ability of trapping the peroxy radical in the ferulic acid group was stronger than that of the α-tocopherol group. The mechanism of antioxidant activity of ferulic acid is primarily in quenching peroxy radicals in linoleic acid to prevent further generation of free radicals.

Table 7. Production of hydroperoxides of control, α-tocopherol, and ferulic acid treatment after 120 and 200 minutes of oxidation (treatment component : linoleic acid molar ratio = 1 : 250)

Products	13HPODE(c,t)		13HPODE(t,t)		9HPODE(t,c)		9HPODE(t,t)	
Time (min)	120	200	120	200	120	200	120	200
Control	225.7±36.1	316.2±56.9	517.9±77.7	950.4±76.0	71.8± 8.6	134.1± 6.7	185.5±31.5	370.8±14.8
α-Tocopherol	78.7± 7.9	119.7± 9.6	41.0± 3.3	54.2± 3.8	50.7± 6.1	78.7± 6.3	26.8± 2.1	34.6± 2.8
Ferulic acid	48.9± 3.9	70.5± 7.8	151.0±22.6	244.8±39.2	24.7± 1.5	61.0± 7.3	60.8± 4.9	100.2±15.0

4.3.6. Antioxidant activity of α-tocopherol at different ratios to linoleic acid

The rates of hydroperoxide production in linoleic acid with α-tocopherol (α-tocopherol : linoleic acid molar ratio = 1:100, 1:250, and 1:500) are shown in Figure 23(a), 23(b), 23(c) respectively. Antioxidant activity of α-tocopherol was indicated by significantly lower rates of hydroperoxide production. In contrast to other components that reduced the production of hydroperoxides as the antioxidant ratio increased, α-tocopherol did not maintain the lowest rate of production of hydroperoxides when the molar ratio was increased to 1:100. This suggests that increasing concentration of α-tocopherol in linoleic acid above a certain level did not increase antioxidant activity further and even reduced its activity. Jung and Min (1990) reported that tocopherols had significant prooxidant effects at higher concentration. Figure 24 shows the increased concentration of individual hydroperoxide in the linoleic acid model after 200 min with three different ratios of α-tocopherol. The high rate of hydroperoxide production at a higher ratio of α-tocopherol was due primarily to the formation of hydroperoxides containing *cis,trans* or *trans,cis* structure. At the same time, hydroperoxides containing *trans,trans* were extensively inhibited by α-tocopherol. Therefore, high concentration of α-tocopherol still demonstrated scavenging of singlet oxygen to prevent formation of *trans,trans* hydroperoxides but appeared to elicit prooxidant activity by accelerating free radical reactions that produce *cis,trans* and *trans,cis* hydroperoxides.

4.3.7. Antioxidant activity of ferulic acid at different ratios to linoleic acid

Ferulic acid evidenced its antioxidant activity with all three molar ratios (ferulic acid : linoleic acid = 1:100, 1:250, and 1:500) by lowering production of hydroperoxides (Figure 23(a), 23(b), and 23(c)). The production of hydroperoxides with ferulic acid at a ratio of 1:100 was significantly lower than that with the ratio of 1:250 or 1:500. The concentration of hydroperoxides containing *trans,trans* was significantly higher than that of hydroperoxides containing *cis,trans* or *trans,cis* at all three ratios. This supported the mechanism of antioxidant activity where ferulic acid primarily reduces peroxy radical and not by scavenging singlet oxygen, as was the case with α-tocopherol. Ferulic acid inhibits formation of hydroperoxides from peroxy free radicals more efficiency than that of hydroperoxides containing *trans,trans*, which are mostly produced by singlet oxygen attack. Marinova and Yanishlieva (1992) investigated the antioxidant activity of ferulic acid on purified lard triacylglycerol, at 25, 50, 75, and 100°C. The antioxidant activity of ferulic acid remained constant with increasing temperature. Oxidation in lipid with added ferulic acid as antioxidant may largely depend on singlet oxygen from the environment. Ferulic acid is a type of phenolic acid in which antioxidant activity is dependent on the number of hydroxylations (HO-) in the phenolic ring (Cuvelier et al., 1992; Pratt, 1992). Chimi et al. (1991) reported that the rates of reactions of aroxyl radical (produced after phenolic acid gives up a hydrogen proton to peroxy radical) and other radicals, which produce non-radical products, exceeded the rates of reaction of aroxyl radical and other non-radicals, which produce free radicals.

4.3.8. Antioxidant activities of the three major components of γ-oryzanol at different ratios to linoleic acid

Three major components of γ-oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, demonstrated significantly lower rates of hydroperoxide production at the ratios of 1:100 and 1:250 (Figure 23(a) and 23(b)). This observation indicated that the three components of γ-oryzanol possess antioxidant activity and supported the observation of antioxidant activity of γ-oryzanol in previous studies (Duve and White, 1991; Sonntag, 1979). Antioxidant activities of these components decreased significantly with decreasing concentration in the linoleic acid model.

Figures 25, 26, and 27 show the chromatograms of hydroperoxides and hydroxides of linoleic acid with each of the three components of γ-oryzanol (component : linoleic acid molar ratio = 1:250) at different oxidation times (0, 120, and 200 min). The profiles of concentration changes of individual hydroperoxide in linoleic acid models with each of the three components were similar to those with ferulic acid. The concentrations of hydroperoxides containing *trans,trans* increased much more than did the concentrations of hydroperoxides containing *trans,cis* and *cis,trans*. This suggests that the mechanism of antioxidation of components of γ-oryzanol was the same as that of ferulic acid. The antioxidant activity of these components relied on the phenolic hydroxyl group in the ferulate portion of their overall structure. Cuvelier et al. (1992) and Pratt (1992) reported that other components of phenolic acid compounds are important factors that affect antioxidant activity. Ohta et al. (1994) found that antioxidant activities of ferulic acid sugar esters were stronger than that of free ferulic

acid in a microsomal lipid peroxidation system. In the linoleic acid model of this study, free ferulic acid had higher activity than the three ferulic acid sterol esters did at the three different ratios, especially at a lower concentration molar ratio (1:500). The triterpene portion of γ-oryzanol may affect its antioxidant activity.

Sterol (triterpene), a part of these components, was reported to have antipolymerization activity in heated oils (Tian and White, 1994; White and Armstrong, 1986; Yan and White, 1990). The side chain of sterol is important for the antipolymerization activity (Tian and White, 1994). Takagi and Iida (1980) reported that the antioxidant activity of canary seed extract (Phalaris canariensis) was due to various sterols such as gramisterol, cycloartenol, sitosterol, campesterol, and triterpene alcohol esters of caffeic acid. Since the hydroxyl of sterol, which would potentate antioxidant activity in the sterol portion, is not free in γ-oryzanol, the sterol component of γ-oryzanol may not contribute to antioxidant activity.

The rate of production of hydroperoxides in the linoleic acid model with each of the three major components of γ-oryzanol was significantly higher than that of α-tocopherol at ratios of 1:250 and 1:500. However, at a ratio of 1:100, the rates of production of hydroperoxides of these components were lower than that of α-tocopherol. Therefore, antioxidant activities of the three components of γ-oryzanol were significantly lower than that of α-tocopherol or ferulic acid at lower concentrations in the linoleic acid model.

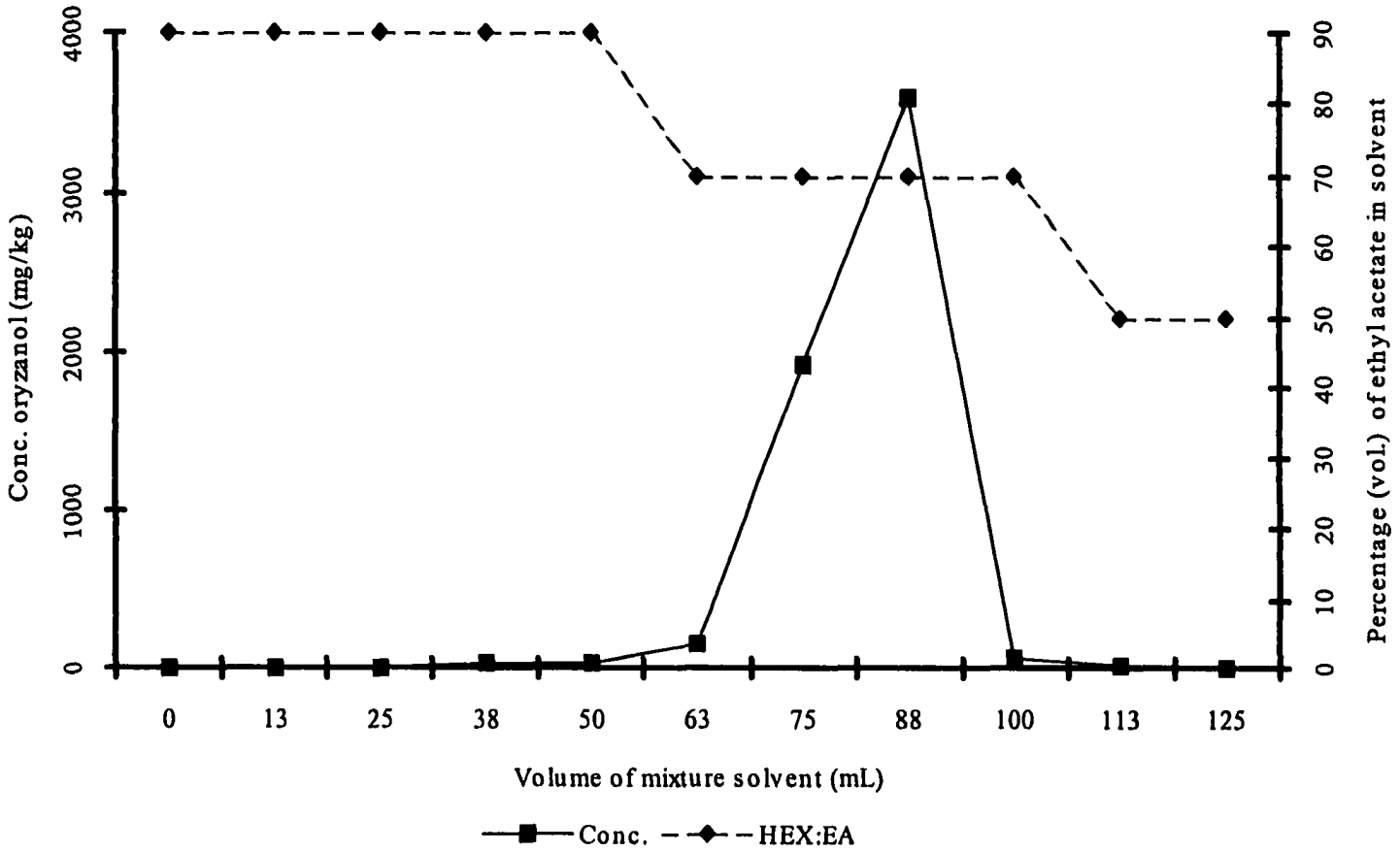


Figure 5. Chromatogram of crude rice bran oil in the low pressure silica column.

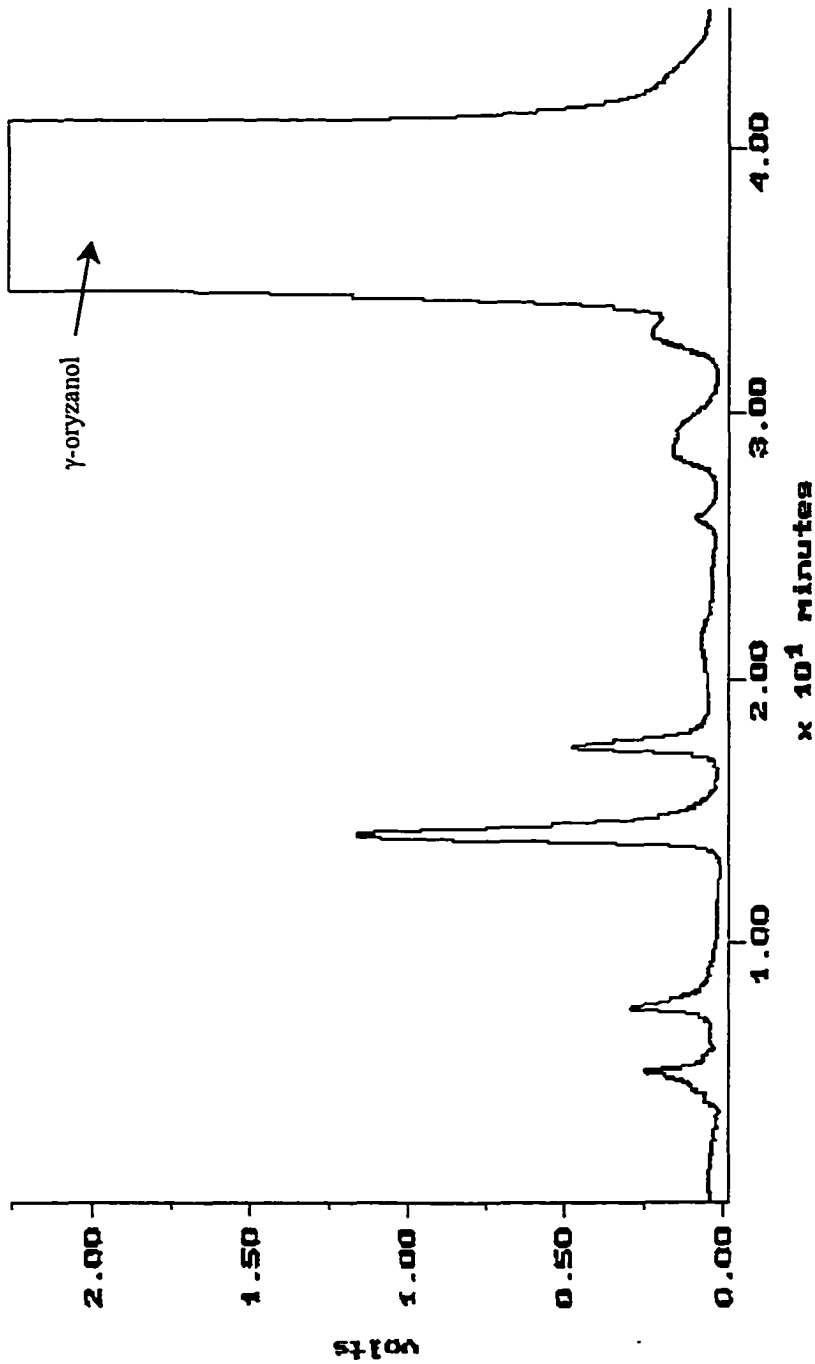


Figure 6. Chromatogram of semi-purified γ-oryzanol in the normal phase preparative HPLC.

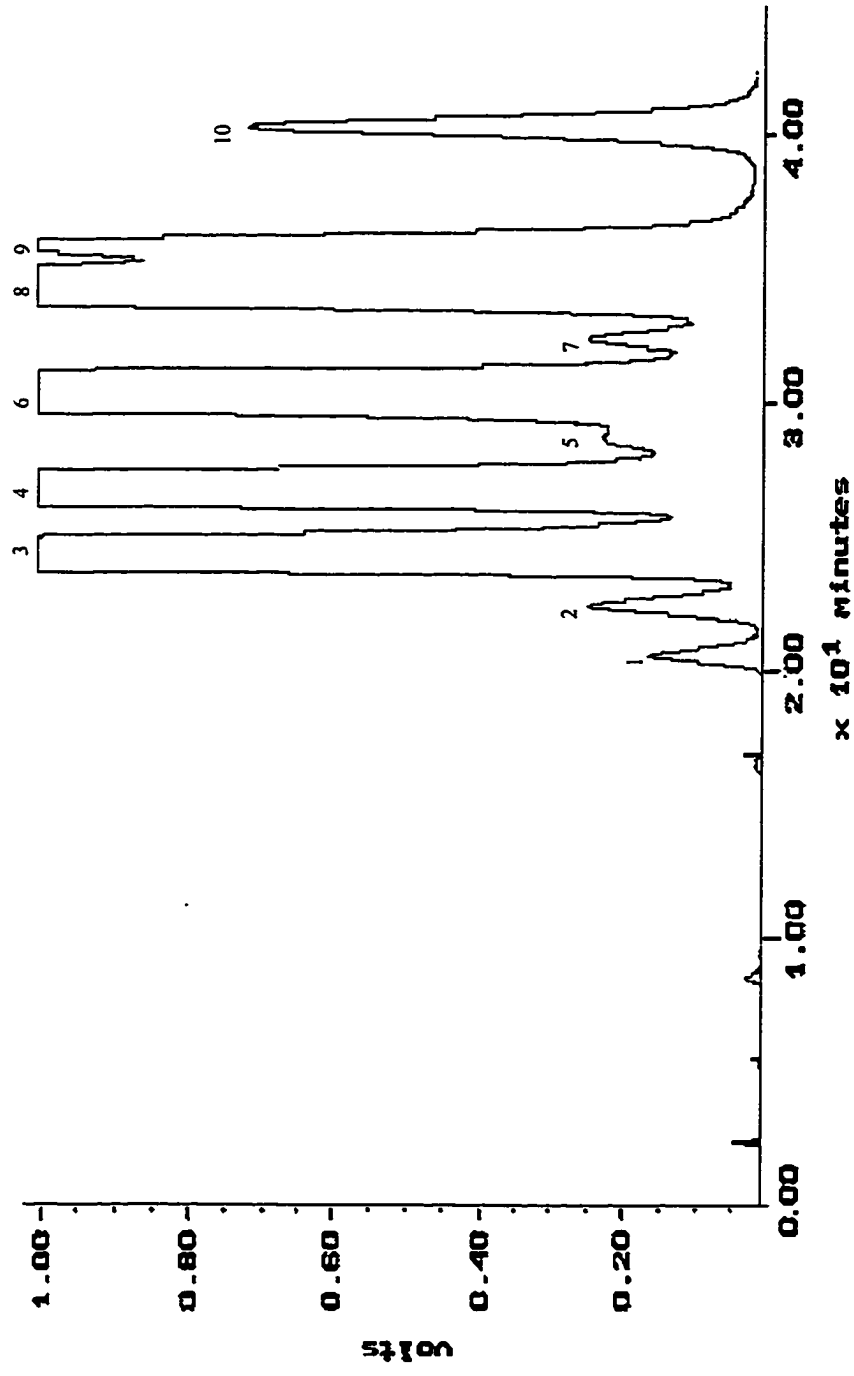


Figure 7. Chromatogram of high purity γ-oryzanol in the analytical reverse phase HPLC for collecting fraction.

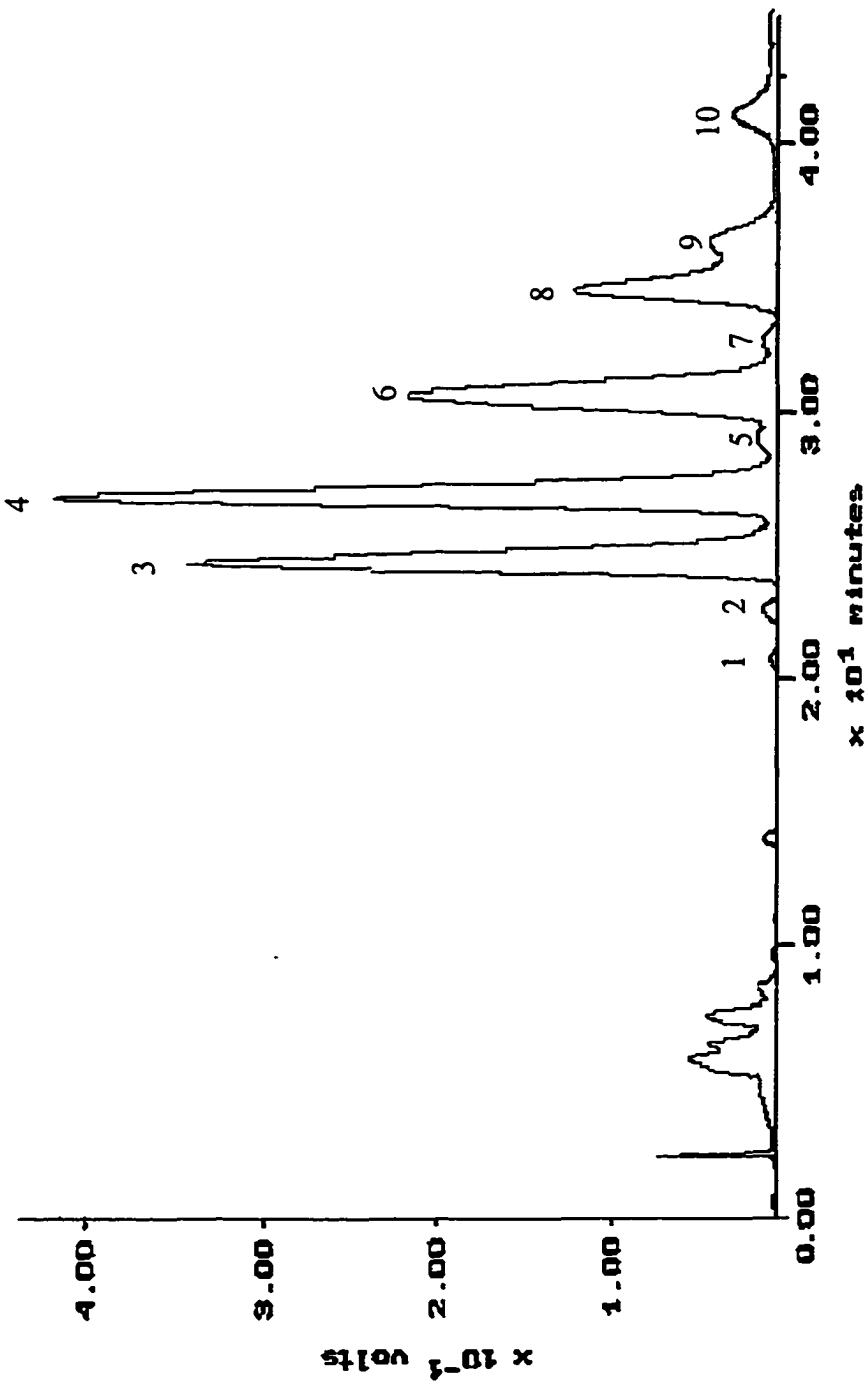


Figure 8. Chromatogram of crude rice bran oil in the analytical reverse phase HPLC.

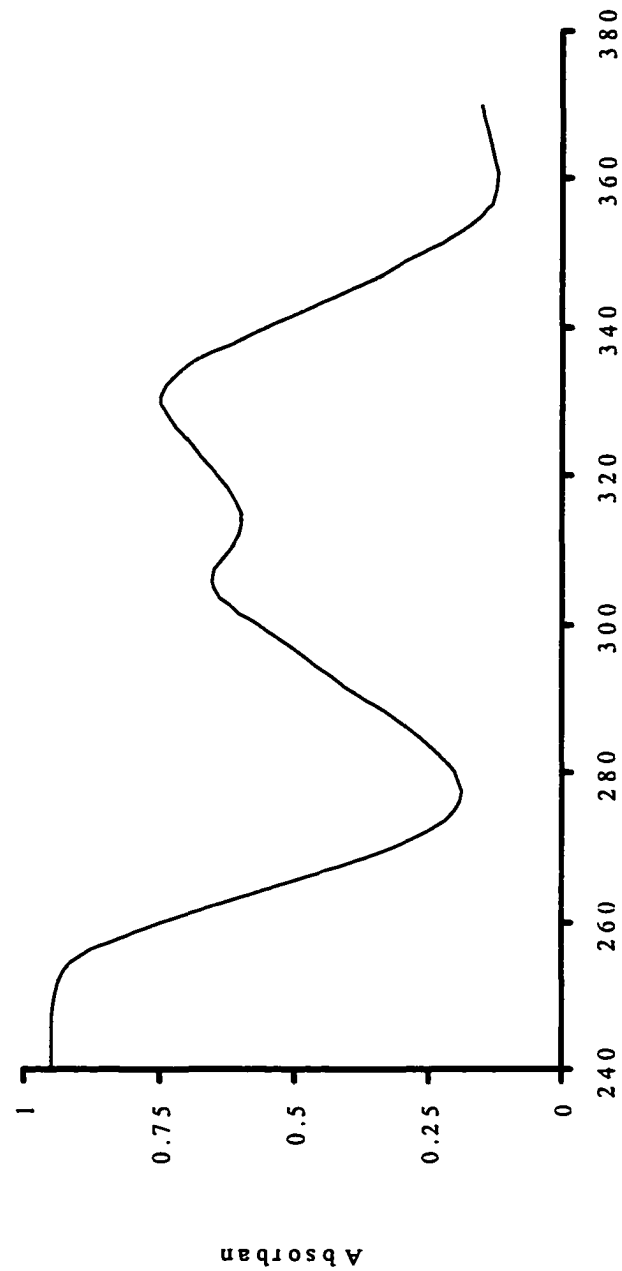


Figure 9. Spectrum of absorption of sitostanyl ferulate.

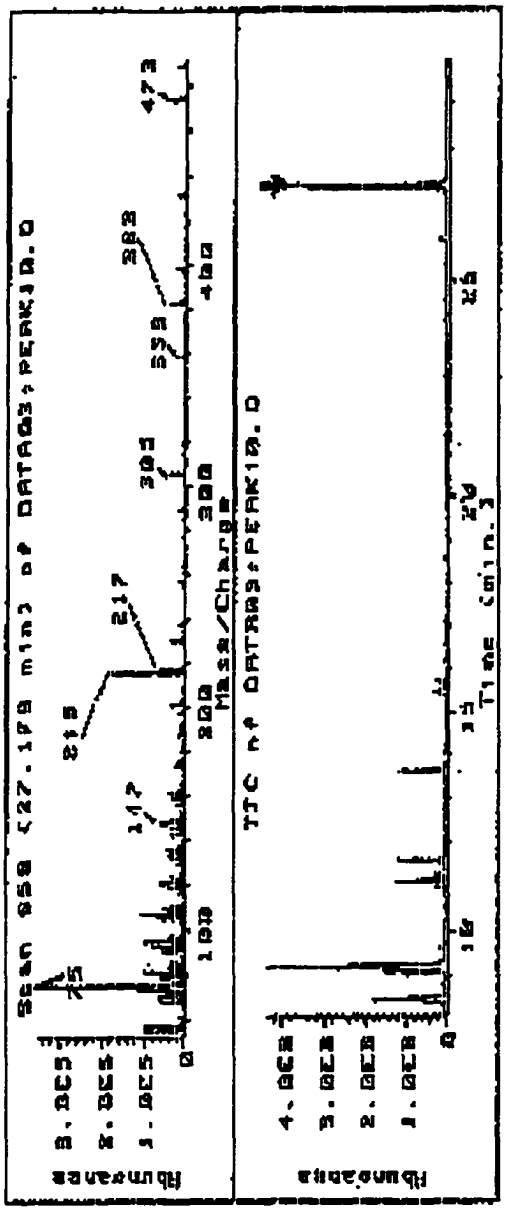
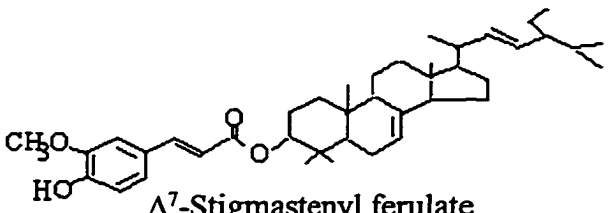


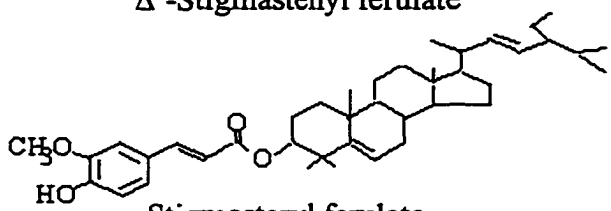
Figure 10. Mass spectra and total ion chromatogram of sitostanol-TMS in GC/MS.

1)



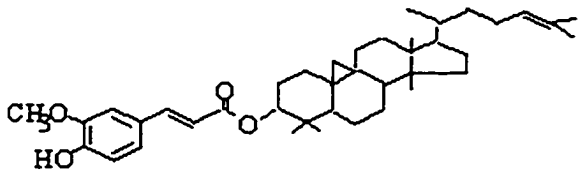
Δ^7-Stigmastenyl ferulate

2)



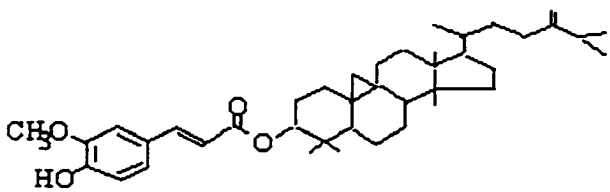
Stigmasteryl ferulate

3)



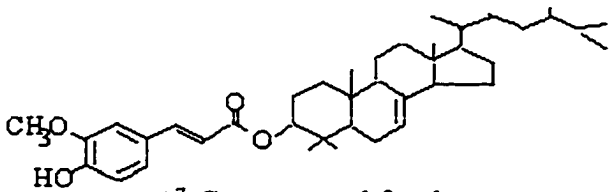
Cycloartenyl ferulate

4)



24-Methylene cycloartanyl ferulate

5)



Δ^7-Campestenyl ferulate

Figure 11(a). Molecular structures of identified components of γ-orzyanol.

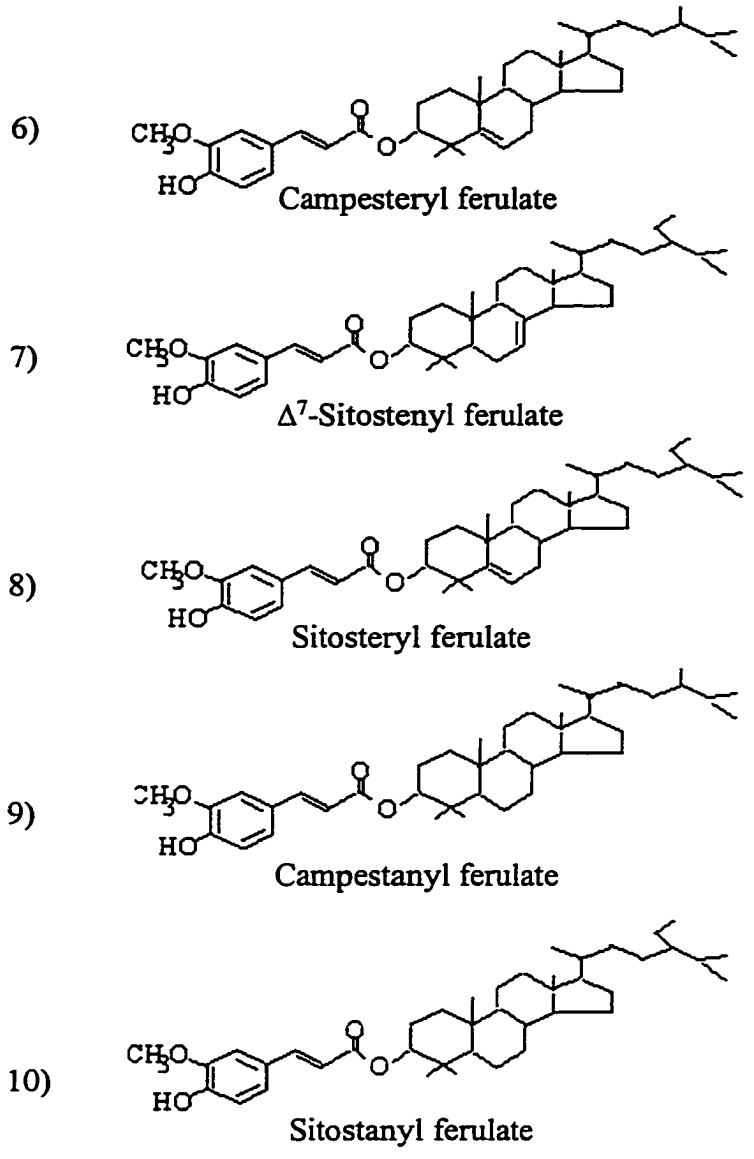


Figure 11(b). Molecular structures of identified components of γ-orzyanol.

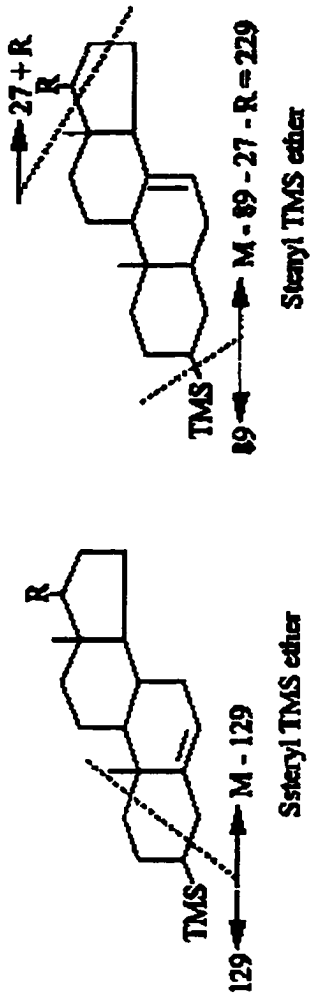


Figure 12. The possible manner of formation of m/z 129 in sterol-TMS and m/z 229 in Δ^7-stenol-TMS.

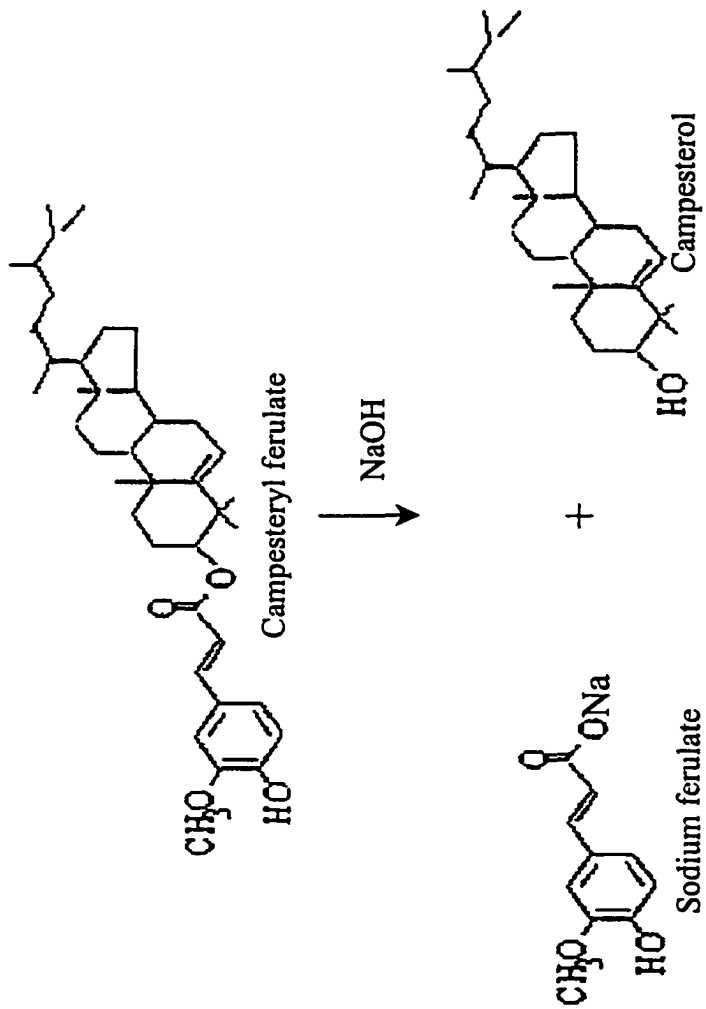
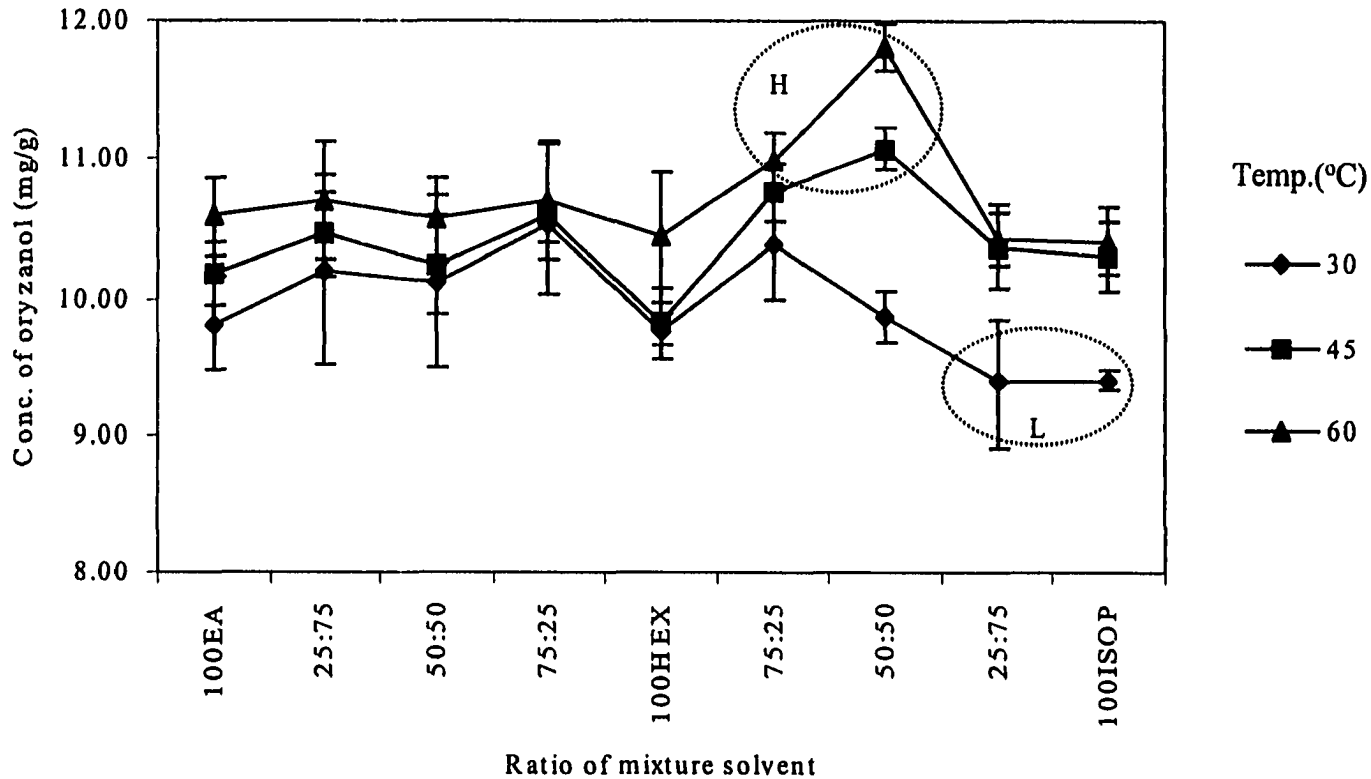


Figure 13. Hydrolysis of campesteryl feruate under alkali condition.



H--Significantly high group; L--significantly low group; EA--Ethyl acetate; HEX--Hexane; ISOP--Isopropanol.

Figure 14. Concentration of γ-oryzanol in extracted oil using different solvent ratios at 30, 45, and 60°C for 60 min.

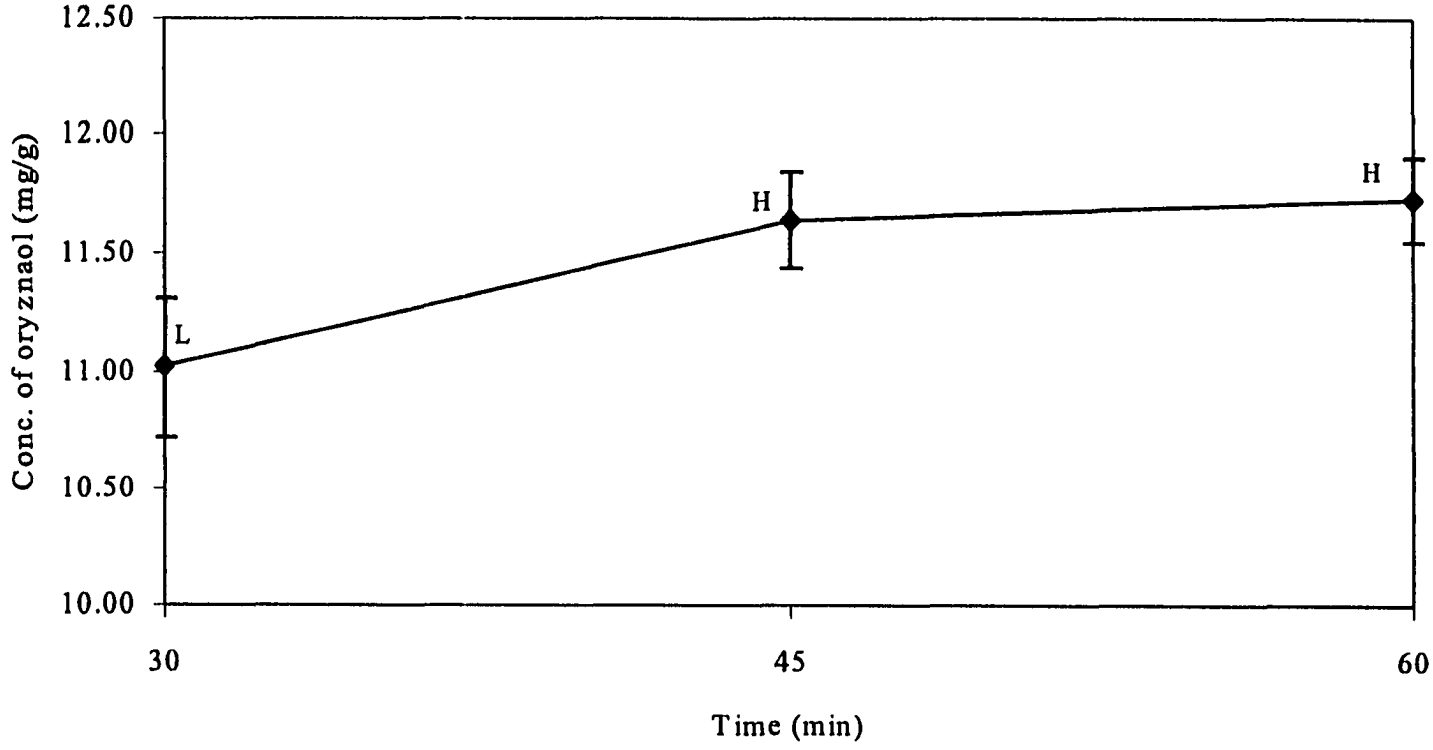
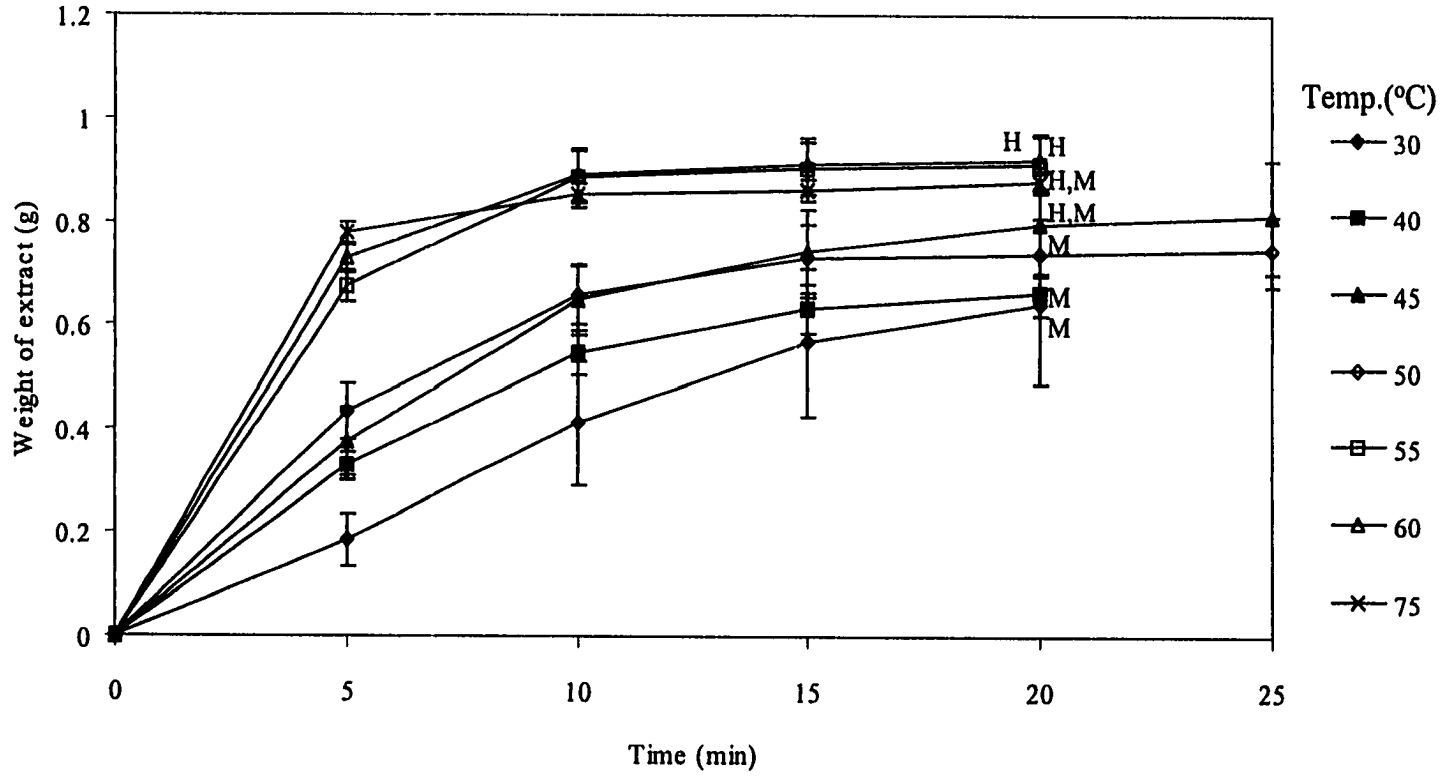
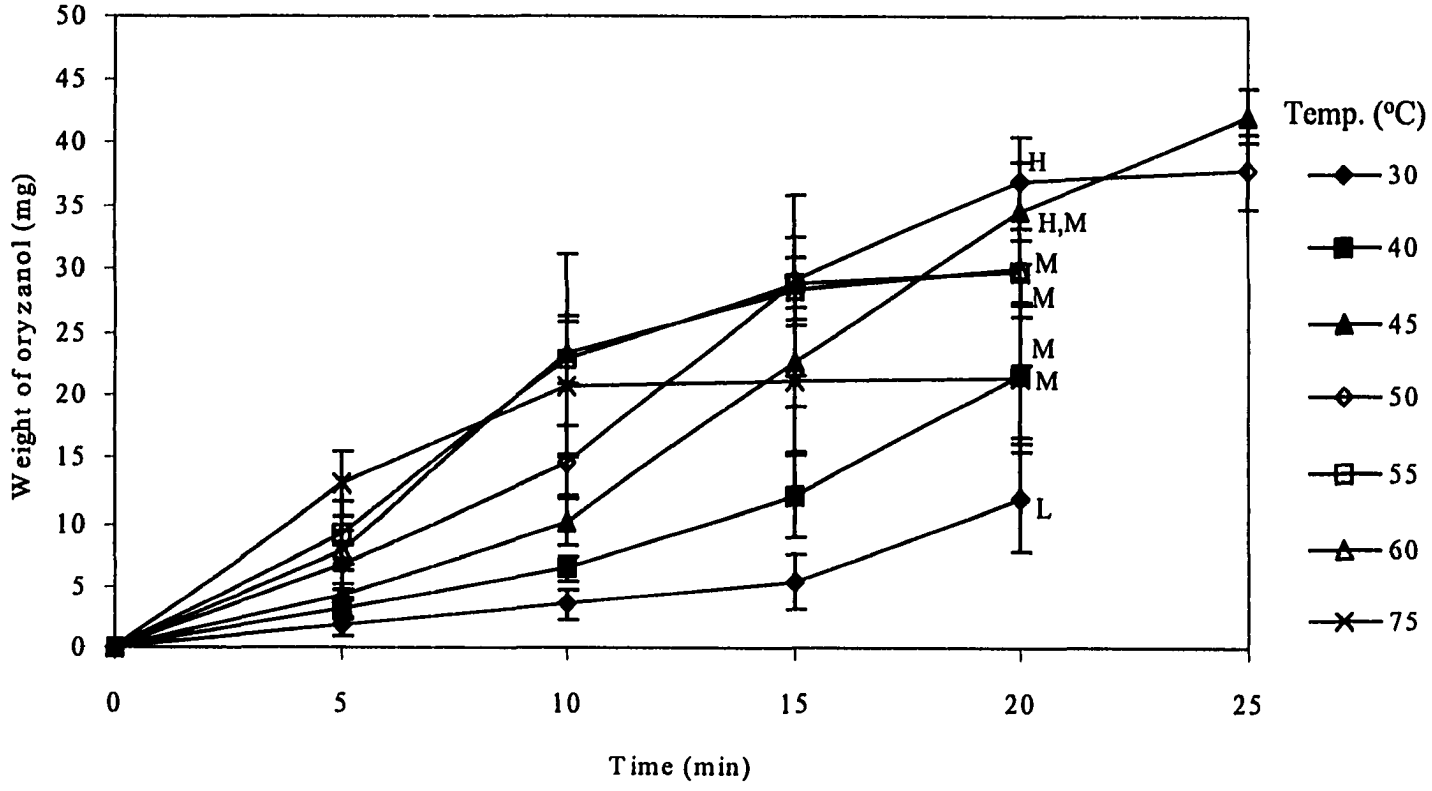


Figure 15. Concentration of γ-oryzanol in extracted oil using the solvent mixture hexane: isopropanol (50 :50, v/v) after 30, 45, and 60 min at 60°C.



Significant difference ($P<0.05$) is expressed by different letters.

Figure 16. Yield of extract of SFE at different extraction times and temperatures.



Significant difference ($P<0.05$) is expressed by different letters.

Figure 17. Yield of γ-oryzanol in extract of SFE at different times and temperatures.

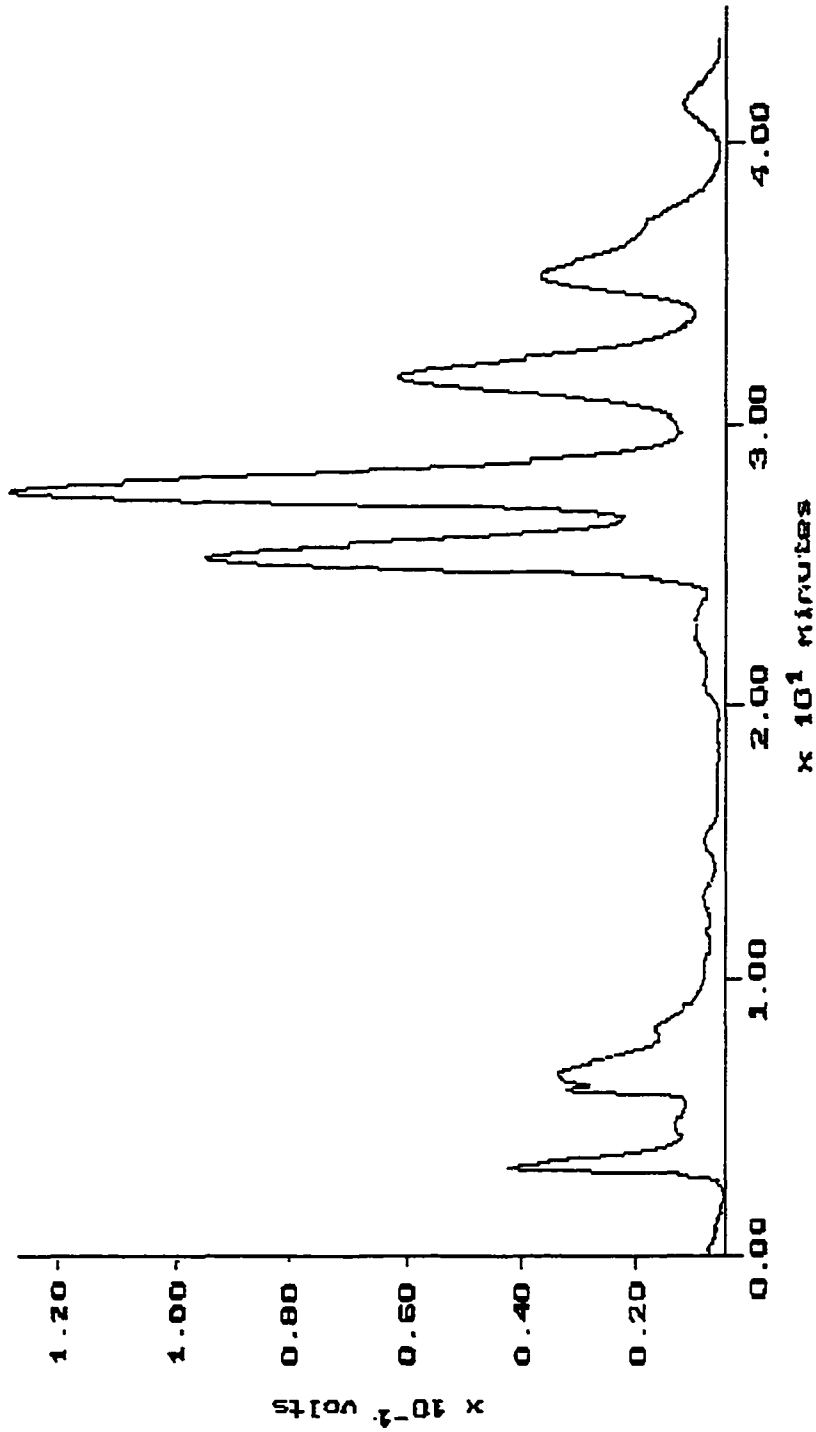
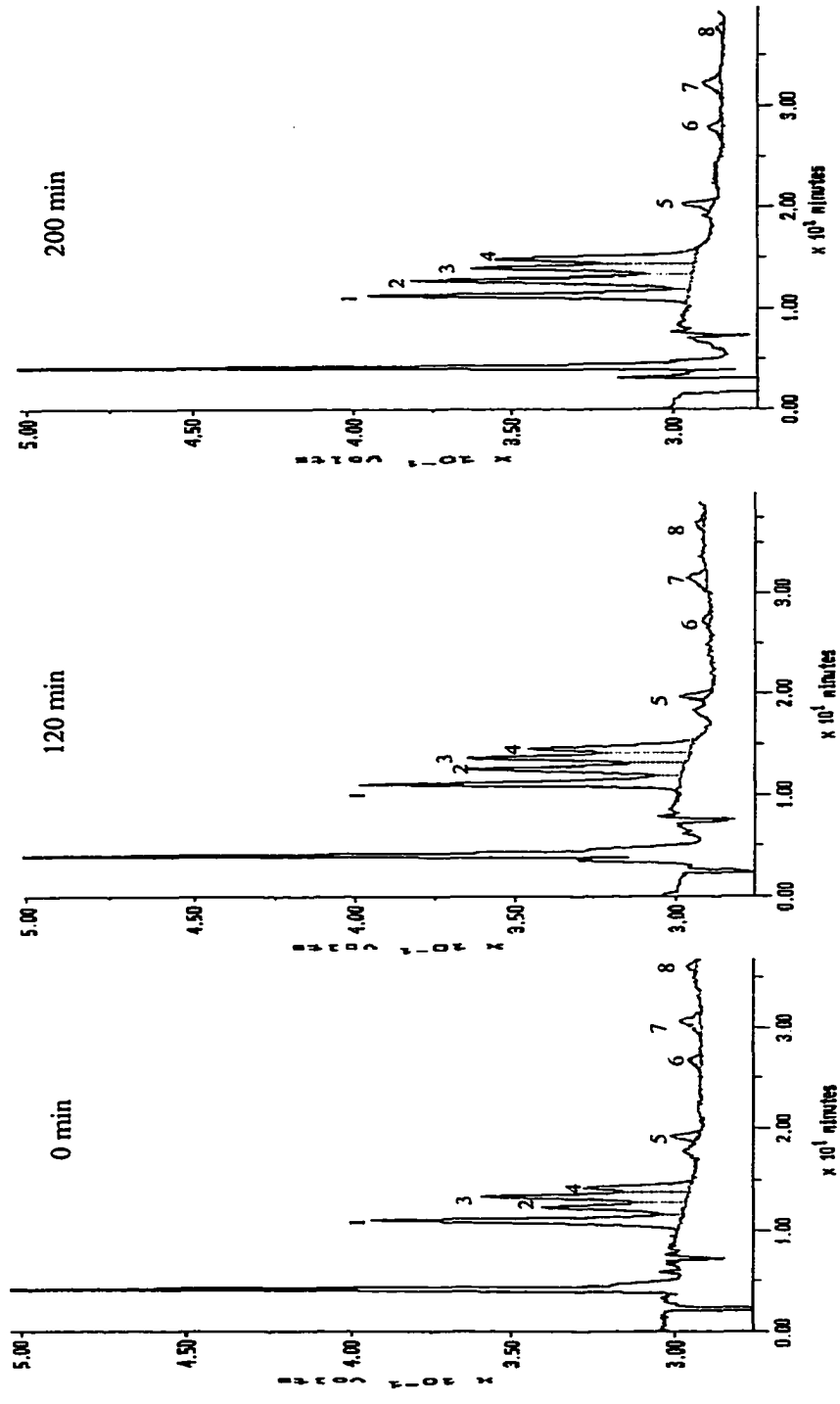


Figure 18. Chromatogram of extract of SFE in the reverse phase preparative HPLC.



1--13HPODE(c,t); 2--13HPODE(t,t); 3--9HPODE(t,c); 4--9HPODE(t,t); 5-- 13HODE(c,t); 6--13HODE(t,t); 7--9HODE(t,c); 8--9HODE(t,t).

Figure 19. Chromatograms of hydroperoxides and hydroxides of linoleic acid at different oxidation times.

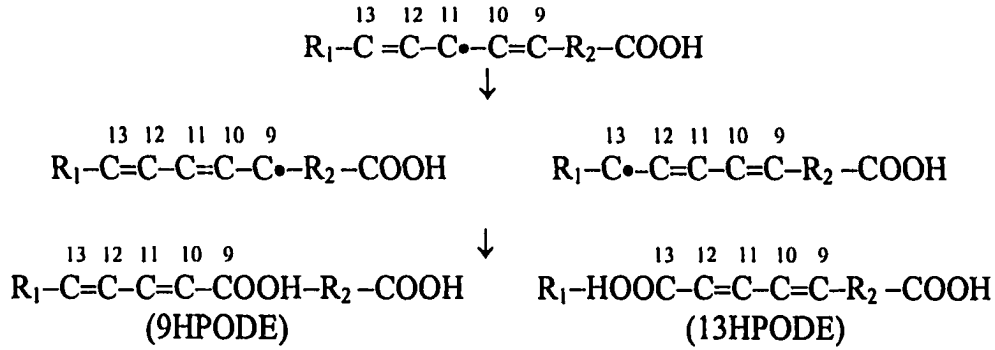


Figure 20. Mechanism of formation of hydroperoxides of linoleic acid during autooxidation

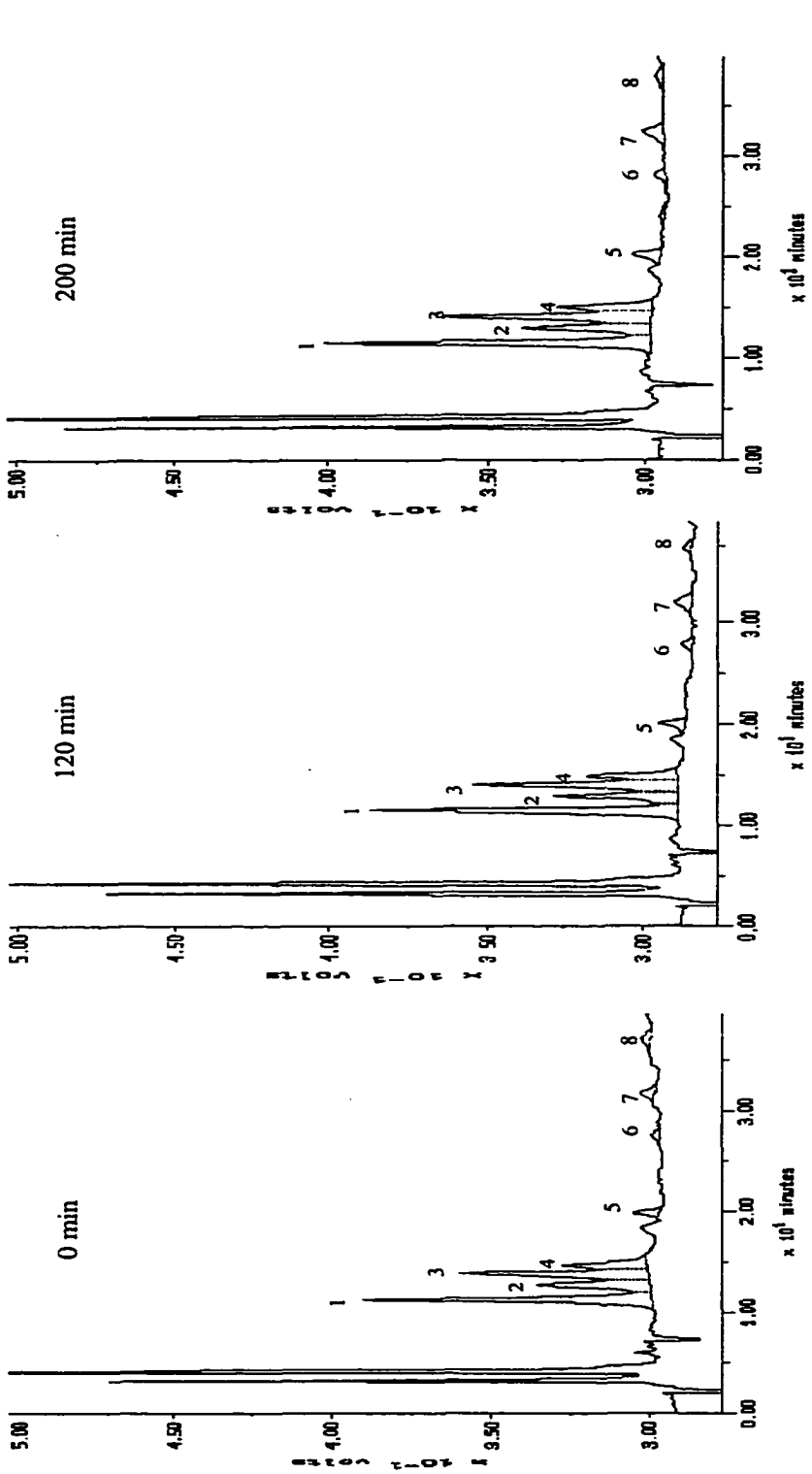
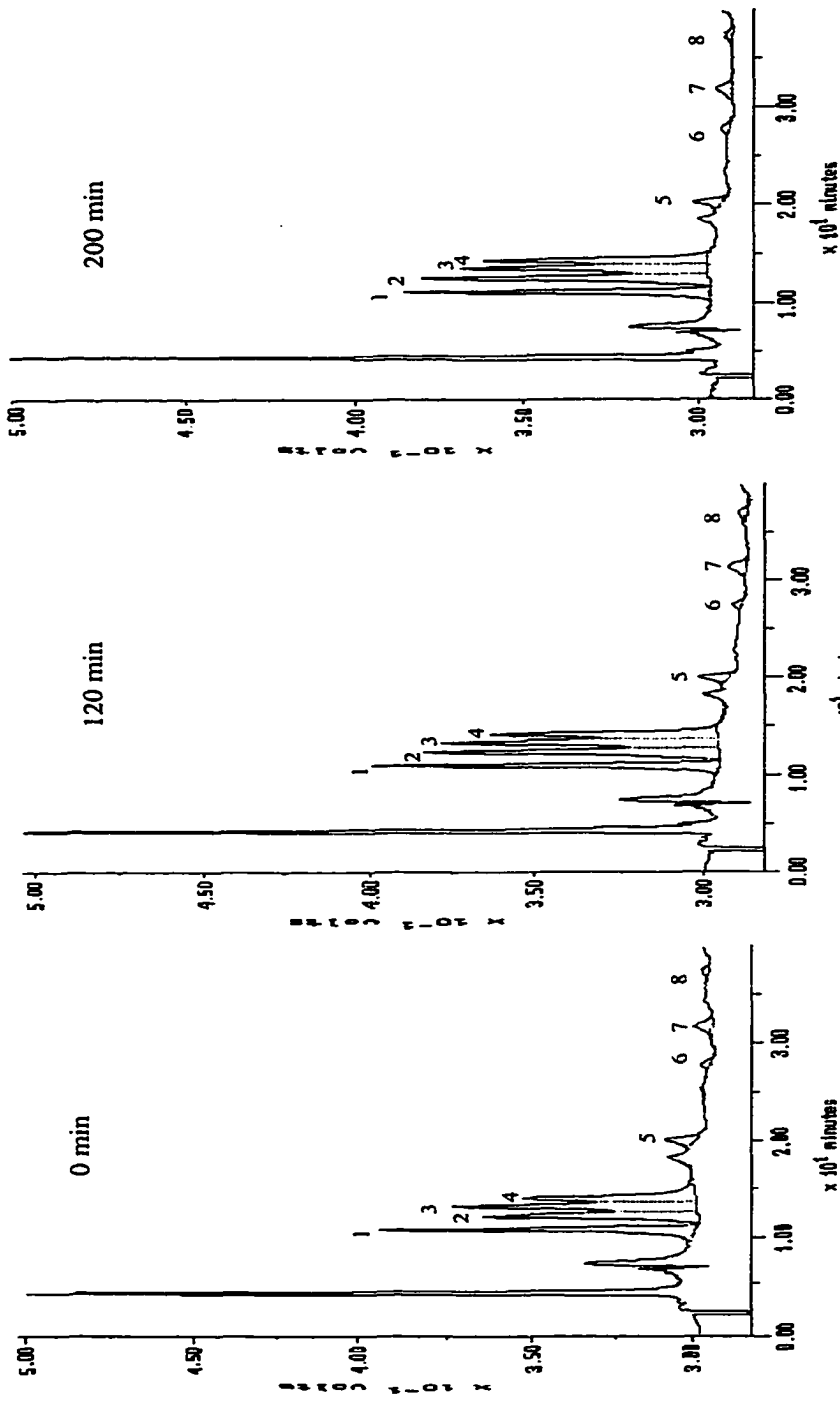
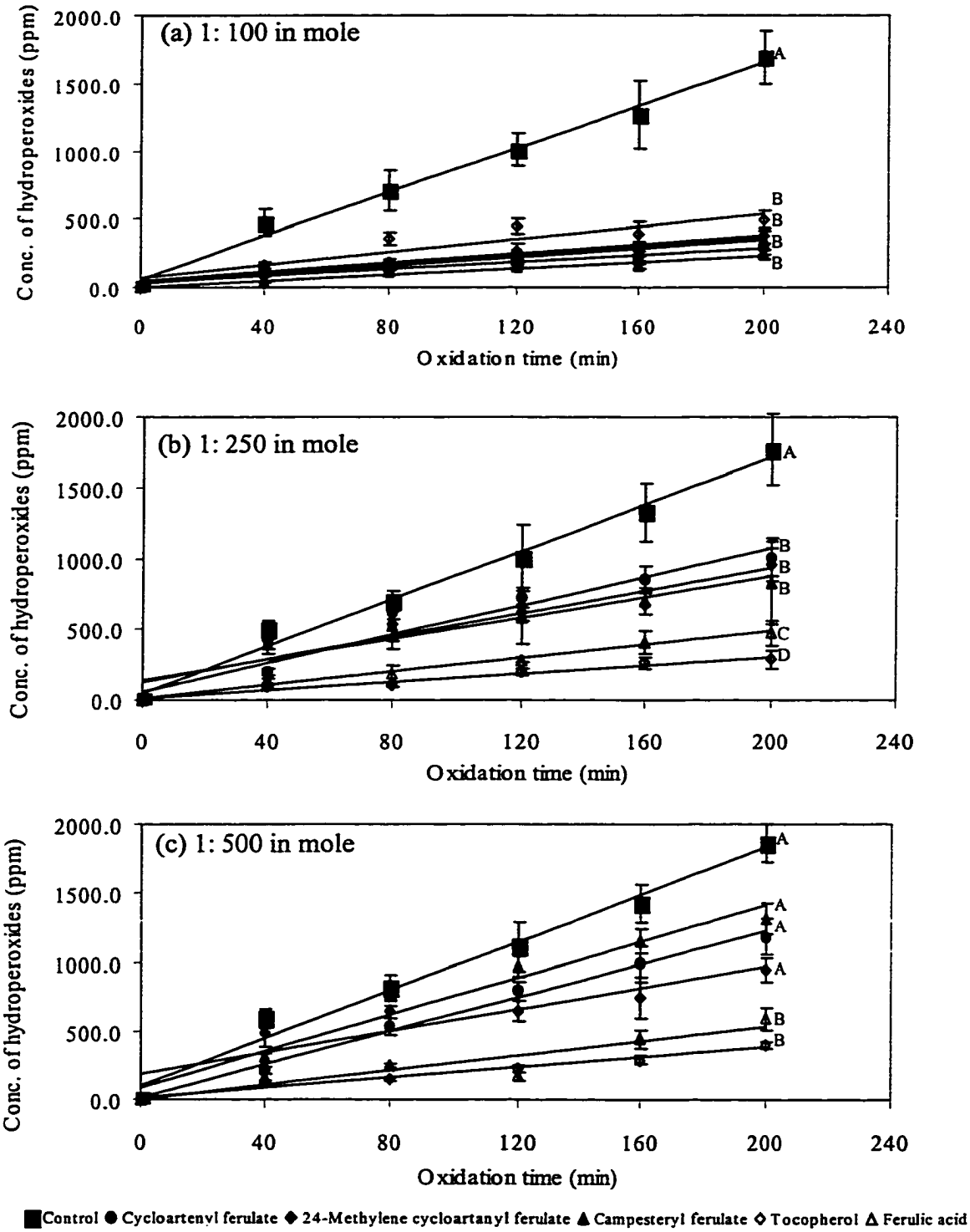


Figure 21. Chromatograms of hydroperoxides and hydroxides of linoleic acid with α-tocopherol (α-tocopherol : linoleic acid molar ratio = 1 : 250) at different oxidation times.



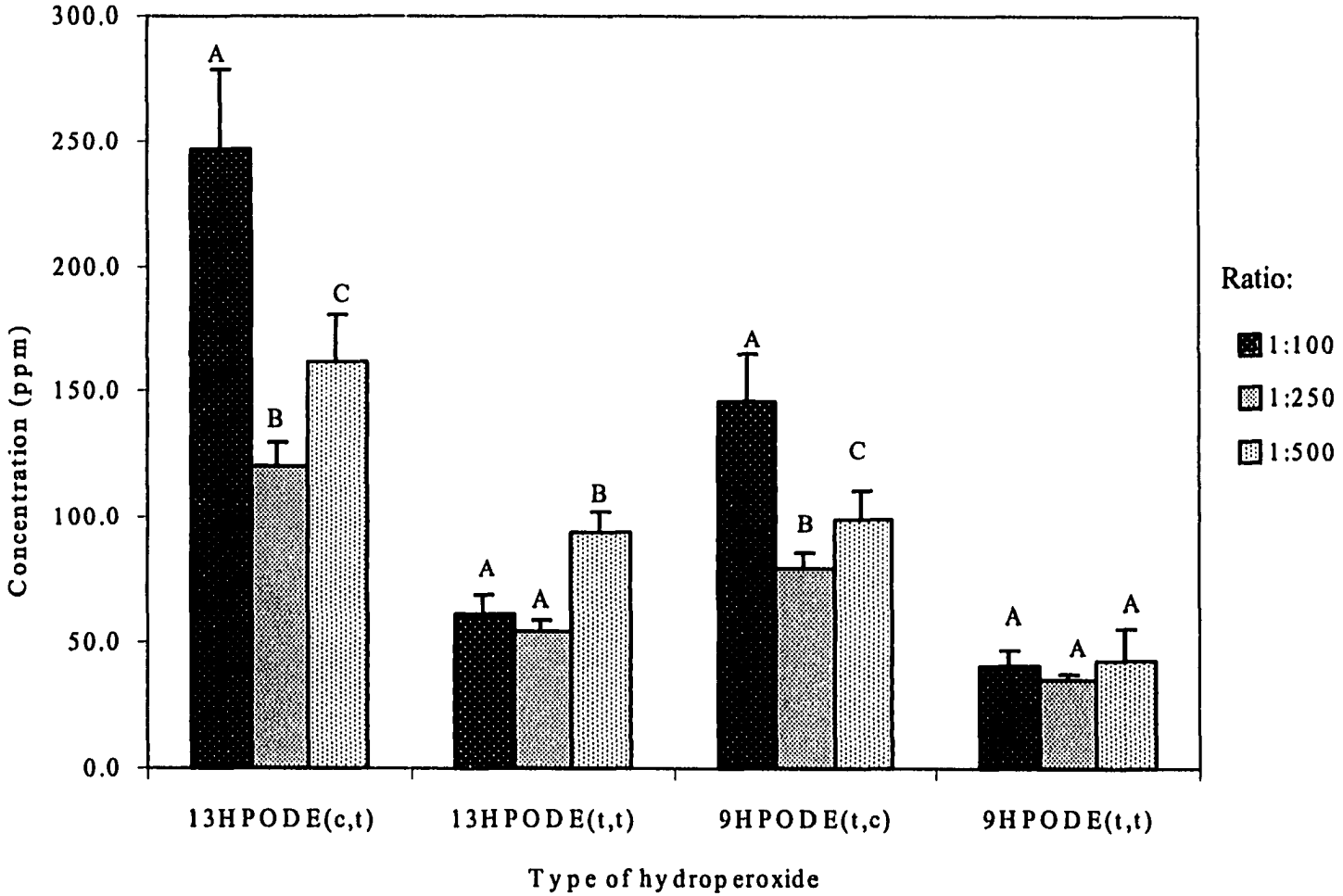
1--13HPODE(c,t); 2--13HPODE(t,t); 3--9HPODE(t,c); 4--9HPODE(t,t); 5-- 13HODE(c,t); 6--13HODE(t,t); 7--9HODE(t,c); 8--9HODE(t,t).

Figure 22. Chromatograms of hydroperoxides and hydroxides of linoleic acid with ferulic acid (ferulic acid : linoleic acid molar ratio = 1 : 250) at different oxidation times.



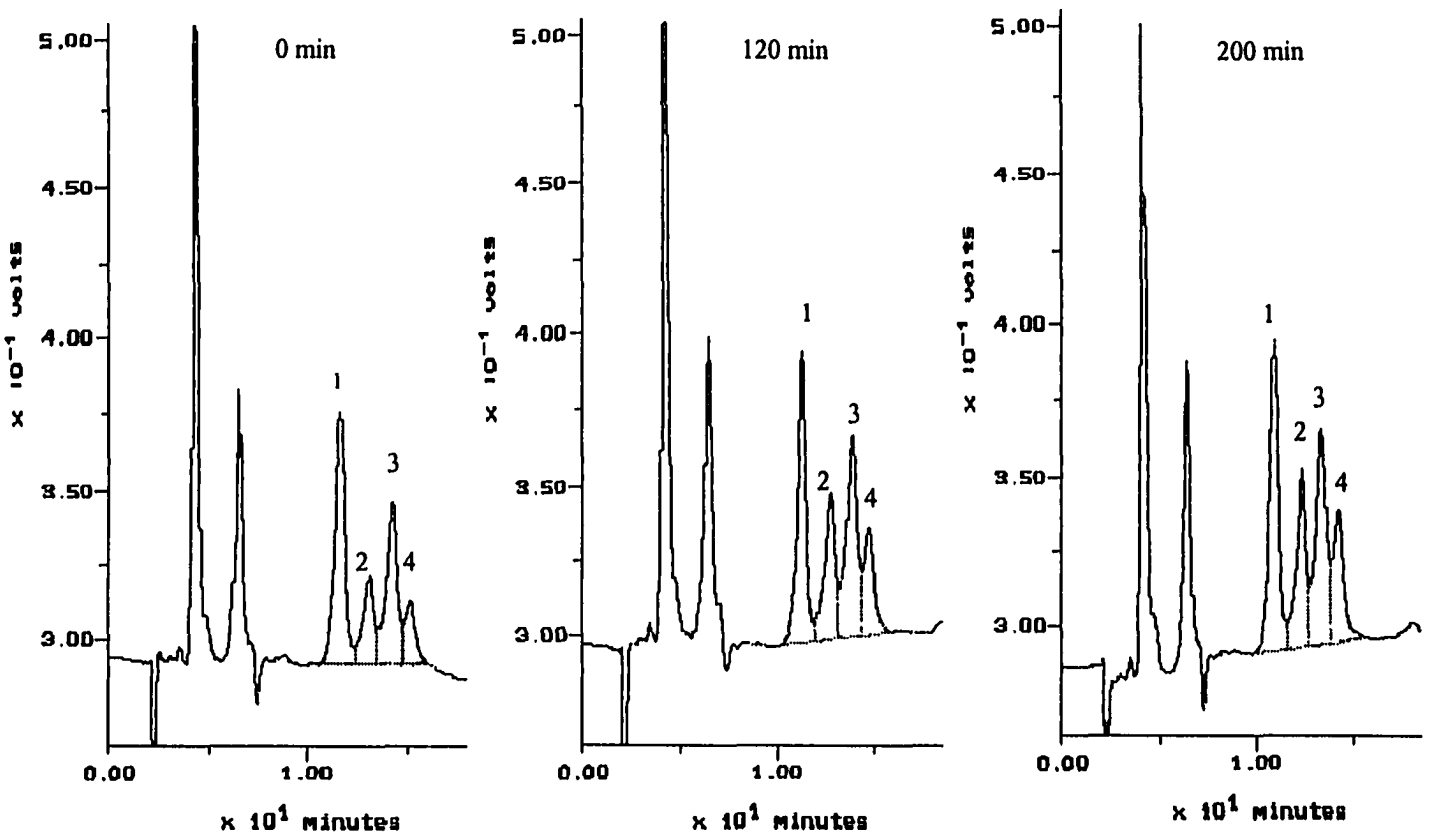
Significant difference in rate ($P<0.05$) is expressed by different letters

Figure 23. Rates of production of total hydroperoxides of linoleic acid with different ratios of component to linoleic acid and control.



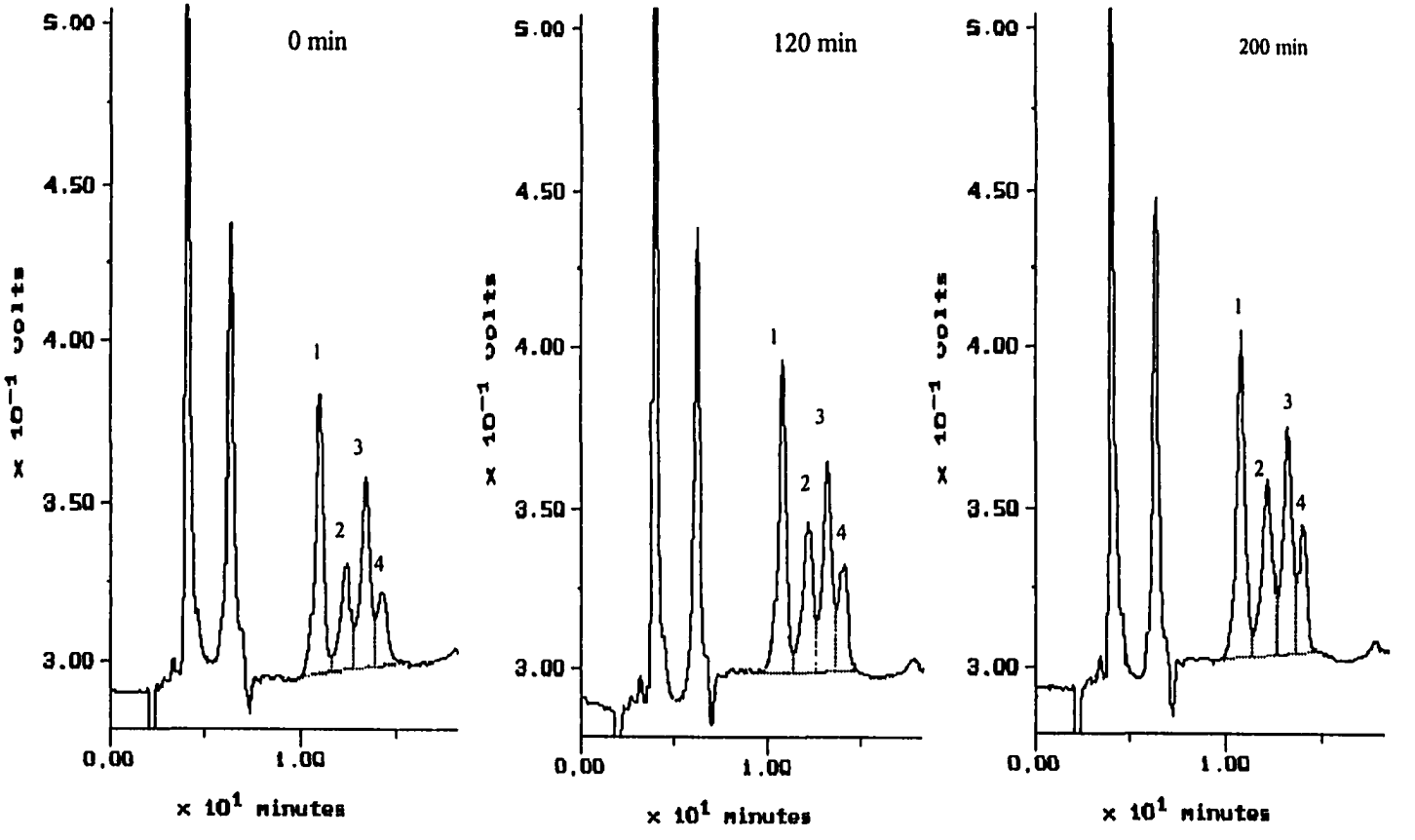
Significant difference ($P<0.05$) is expressed by different letters on the two bars in a cluster.

Figure 24. Production of hydroperoxides of linoleic acid with different molar ratios of α-tocopherol to linoleic acid after 200 min oxidation.



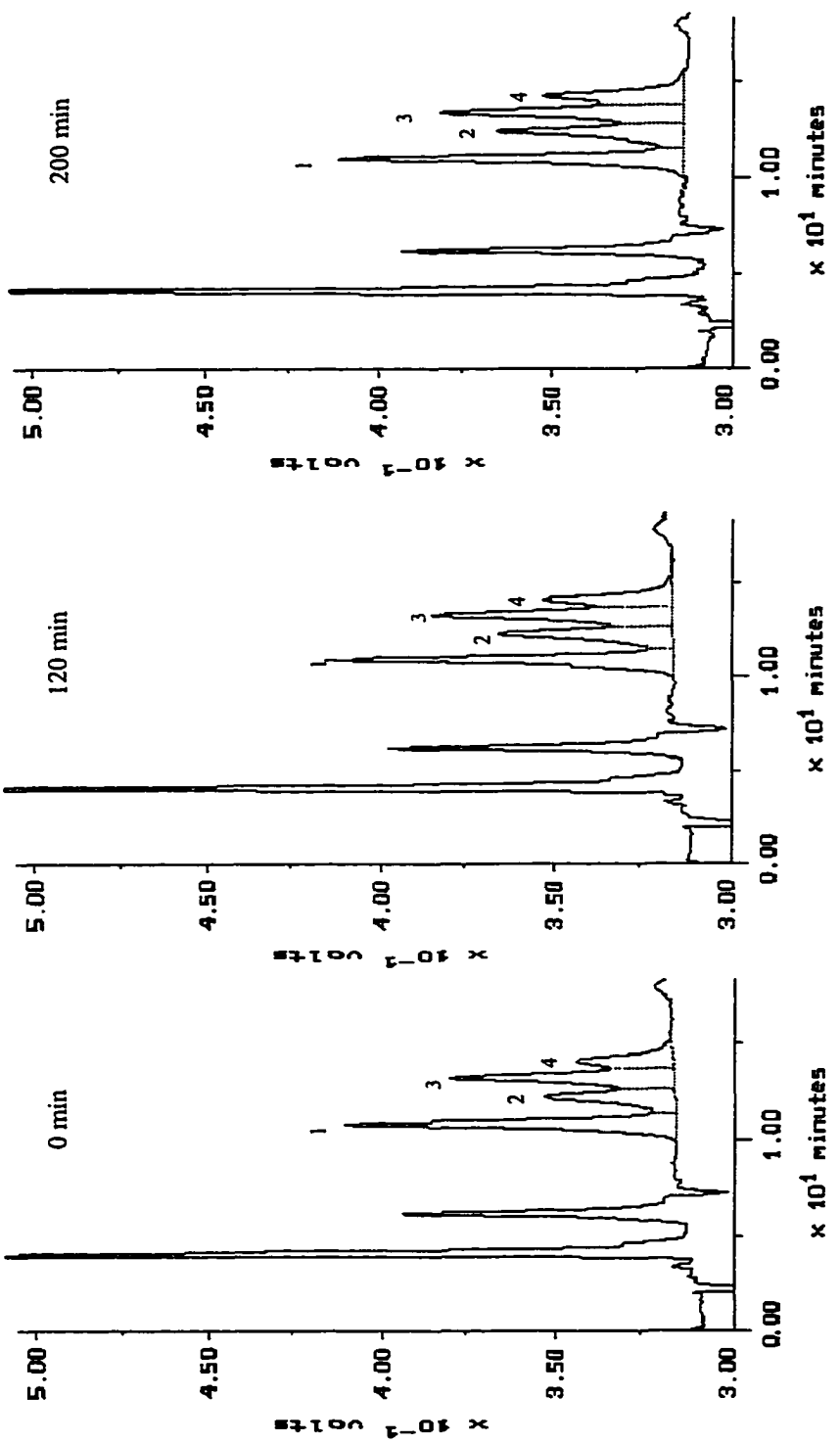
1--13HPODE(c,t); 2--13HPODE(t,t); 3--9HPODE(t,c); 4--9HPODE(t,t).

Figure 25. Chromatograms of hydroperoxides of linoleic acid with cycloartenyl ferulate (cycloartenyl ferulate : linoleic acid molar ratio = 1 : 250) at different oxidation times.



1--13HPODE(c,t); 2--13HPODE(t,t); 3--9HPODE(t,c); 4--9HPODE(t,t).

Figure 26. Chromatograms of hydroperoxides of linoleic acid with 24-methylene cycloartanyl ferulate (24-methylene cycloartanyl ferulate : linoleic acid molar ratio = 1 : 250) at different oxidation times.



1--13HPODE(c,t); 2--13HPODE(t,t); 3--9HPODE(t,c); 4--9HPODE(t,t).

Figure 27. Chromatograms of hydroperoxides of linoleic acid with campesteryl ferulate (campesteryl ferulate : linoleic acid molar ratio = 1 : 250) at different oxidation times.

CHAPTER 5

SUMMARY AND CONCLUSIONS

γ-Oryzanol is a mixture of ferulate esters of triterpene alcohols. Ten ferulate esters of trierpene alcohols, Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campestenyl ferulate, campesteryl ferulate, stigmastenyl ferulate, sitosteryl ferulate, compestanyl ferulate, and sitostanyl ferulate, were identified in γ-oryzanol from rice bran oil. Three of these, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, are major components. They occupy approximately 70% of γ-oryzanol. A reverse phase HPLC method was successfully developed to isolate and quantify each of these ten components.

γ-Oryzanol can be extracted from rice bran using traditional solvent extraction or supercritical fluid extraction. Saponification that usually is more beneficial and used in solvent extraction reduced the yield of γ-oryzanol in half, compared to extraction without saponification. A solvent mix of 50% hexane and 50% isopropanol at 60°C extraction temperature extracted the highest yield of γ-oryzanol from rice bran among solvents of hexane, ethyl acetate, isopropanol, mixtures of hexane and ethyl acetate, and mixtures of hexane and isopropanol. The time of extraction using a solvent mix of 50% hexane and 50% isopropanol at 60°C was 45 to 60 min. Supercritical fluid extraction significantly improved the yield of γ-oryzanol from rice bran. The yield of γ-oryzanol in the supercritical fluid extraction at a temperature of 50°C, pressure 680 atm, and time

25 min was approximately four times higher than that of solvent extraction. Fractionating of extract in the supercritical fluid extraction was successfully used in gaining high concentration of γ-oryzanol in extract. The high concentration (50 - 80%) of γ-oryzanol was obtained by collecting extract between 15 - 20 min under these extraction conditions. This was sixty times purer than that obtained from solvent extraction.

Purification using low pressure chromatography before isolating individual components in preparative scale HPLC was necessary to concentrate γ-oryzanol extracted with solvents because the extract contained high levels of interfering lipids. However, extract with a high concentration of γ-oryzanol obtained from the supercritical fluid extraction could be directly loaded onto the preparative scale HPLC without the need for concentration. This avoided the need for large amounts of solvent and time in solvent extraction and purification. In normal phase preparative scale HPLC, it was not possible to isolate individual components of γ-oryzanol. γ-Oryzanol eluted from the normal phase column as one fraction. In reverse phase preparative scale HPLC, individual components of γ-oryzanol were successfully separated.

A mild oxidation model was implemented for evaluating antioxidant activity of compounds. Linoleic acid in hexane with continuing air input at 37°C proved to be a sensitive and highly specific HPLC quantitative method. Four hydroperoxide isomers of linoleic acid were measured to observe the oxidation status of linoleic acid during oxidation. Also, the mechanism of antioxidant function could be observed by the relative production of *trans,trans* hydroperoxides, and *trans,cis* and *cis,trans*

hydroperoxides. Antioxidant activity via scavenging peroxyl or oxyl free radicals could be seen in a lower production of *trans,cis* or *cis,trans* hydroperoxides and a higher production of *trans,trans* hydroperoxides. Antioxidant activity via scavenging singlet oxygen had the opposite effect. From this linoleic acid model, different antioxidant mechanisms between α-tocopherol and ferulic acid were observed. The mechanism of the three major components of γ-oryzanol was similar to that of ferulic acid based on the changes in each hydroperoxide. The ferulate portion of the three components appeared to be responsible for their antioxidant activities.

The three major components of γ-oryzanol had significant antioxidant activity when mixed with linoleic acid in molar ratios of 1:100 and 1:250; however, antioxidant activities were not presented when the molar ratio decreased to 1:500. Ferulic acid had significant antioxidant activity in all three molar ratios (1:100, 1:250, and 1:500). α-Tocopherol showed significantly stronger antioxidant activity at molar ratios of 1:250 and 1:500 than that of the three components of γ-oryzanol. However, it had the lowest antioxidant activity at a molar ratio of 1:100. This suggests that α-tocopherol may have prooxidant activity at high concentration in the linoleic acid model.

In conclusion, ten ferulate esters of trierpene alcohol, Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campestenyl ferulate, campesteryl ferulate, stigmastenyl ferulate, sitosteryl ferulate, compestanyl ferulate, and sitostanyl ferulate, were identified in γ-oryzanol from rice bran oil. γ-Oryzanol extracted from rice bran using supercritical fluid extraction had significantly higher yield and concentration of γ-oryzanol under optimized conditions,

which could be directly loaded on reverse phase preparative HPLC to isolate individual components of γ-oryzanol. The three major components of γ-oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, had significant antioxidant activities in the linoleic acid oxidation model in molar ratios of 1:100 and 1:250. Their activities were not as high as that of ferulic acid and α-tocopherol in the linoleic acid oxidation model, except for α-tocopherol in the ratio of 1:100.

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VITA

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Food Science

Title of Dissertation: Purification and Antioxidant Properties of Rice Bran γ-Oryzanol Components

Approved:

J. Samuel Godber
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

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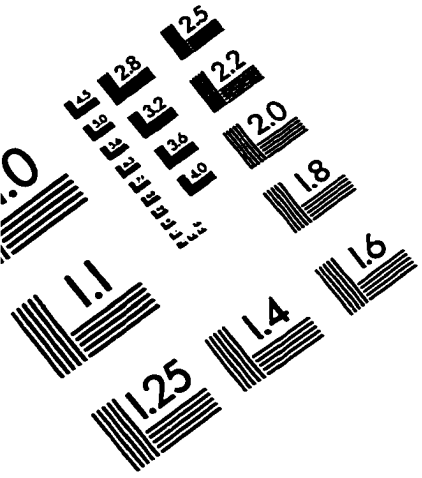
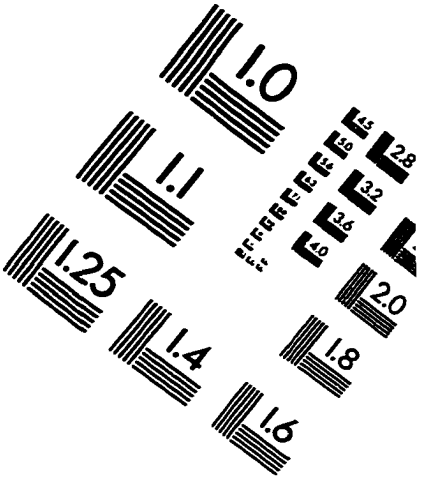
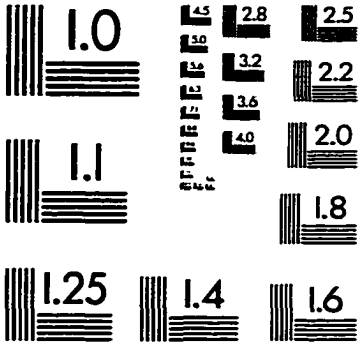
Date of Examination:

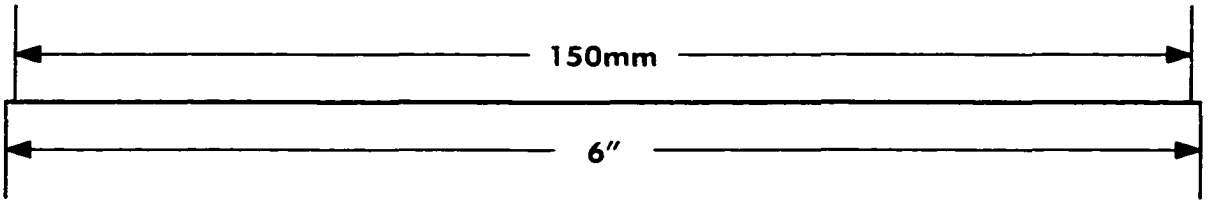
December 10, 1997

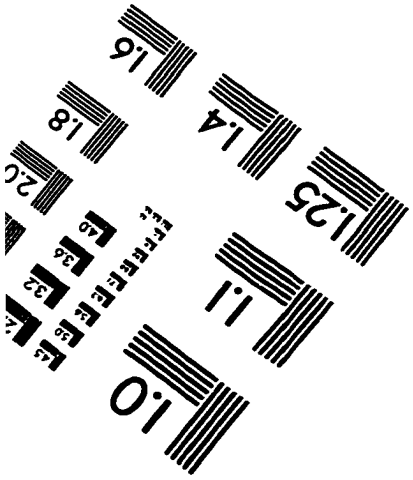
IMAGE EVALUATION TEST TARGET (QA-3)

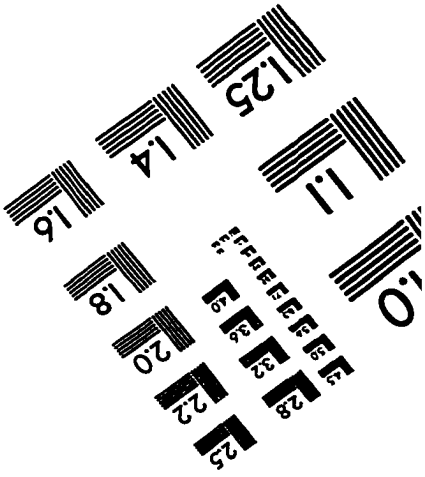












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